

## POLYMER-FACTOR VIII MOIETY CONJUGATES

### CROSS REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of priority to U.S. Provisional Patent Application Serial No. 60/450,578, filed February 26, 2003, which is incorporated herein by reference.

### FIELD OF THE INVENTION

[0002] The present invention relates generally to conjugates comprising a Factor VIII moiety (i.e., a moiety having Factor VIII activity) and a polymer. In addition, the invention relates to compositions comprising the conjugates, methods for synthesizing the conjugates, and methods for treating patients.

### BACKGROUND OF THE INVENTION

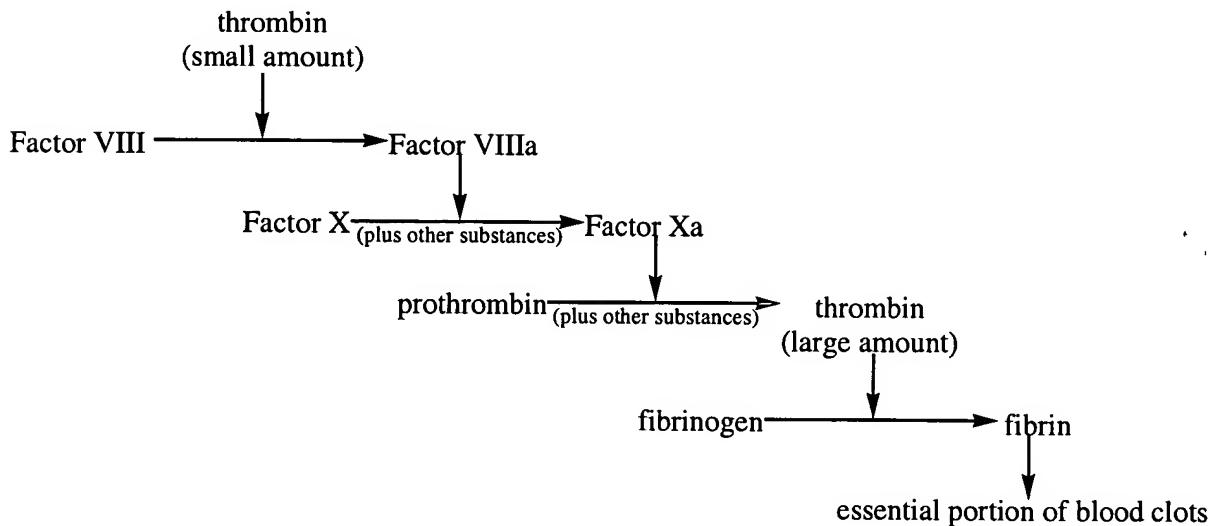
[0003] Hemostasis is the process of arresting the outflow of blood from an injured blood vessel. For mammals, as well as many other organisms, the hemostatic process is critically important for continued survival. Defects in the hemostatic process can result in, for example, the inability to effectively form blood clots that serve to stop the loss of blood following vascular injury. In humans, individuals who suffer from an inability to form blood clots are called hemophiliacs. Of particular concern for hemophiliacs is the life-threatening risk that once started, bleeding will never cease.

[0004] Generally, hemophiliacs lack the ability to produce effective amounts of one or more substances required to form blood clots. For example, hemophiliacs who suffer from hemophilia A (also called "classic hemophilia") have an inability to produce effective levels of Factor VIII (also known as "antihemophilia factor A," "antihemophilic globulin," and "AHG"). Factor VIII is a key component of one of several "cascades" of reactions that result in the formation of blood clots. Critical for the cascade of reactions referred to as the "intrinsic

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pathway," Factor VIII ultimately influences the conversion of fibrinogen into the major component of blood clots, fibrin.

[0005] Although the intrinsic pathway of blood clot formation is relatively complex, the role of Factor VIII can be described briefly. In the presence of relatively small amounts of thrombin (released, for example, by the cells of ruptured tissues), Factor VIII is converted into its activated form known as Factor VIIIa. Factor VIIIa (along with other substances), in turn, activates another factor, Factor X into Factor Xa. Thereafter, Factor Xa (along with other substances) converts prothrombin into thrombin, with the result that a relatively large amount of thrombin is produced over time. Relatively large amounts of thrombin effectively convert fibrinogen into fibrin. Fibrin, in turn, forms the matrix or lattice responsible for the formation of blood clots. Factor VIII's role in the intrinsic pathway of blood clotting is shown schematically below.



[0006] Affecting one or two males for every 10,000 live births in all populations, hemophilia A can result from any one of a variety of mutations of the Factor VIII gene, which is located on the X-chromosome. Depending on the particular mutation, hemophilia A can manifest itself as severe, moderate or mild. Individuals suffering from the severest forms of hemophilia A entirely lack the ability to express active forms of Factor VIII. Clinically, individuals affected with hemophilia A suffer from muscle hemorrhage, joint hemorrhage, easy bruising, and prolonged bleeding from wounds. The mean age of individuals suffering from

hemophilia A without treatment is twenty. Current treatment of hemophilia A involves the infusion of exogenous Factor VIII concentrate collected from human plasma or prepared via recombinant DNA techniques. Because these treatments serve only to supplement the lack of effective levels of Factor VIII, individuals suffering from Factor VIII require regular injections of Factor VIII concentrate throughout their lives.

[0007] Several commercial forms of Factor VIII concentrates are available to provide replacement therapy for patients suffering from hemophilia A. For example, blood-derived Factor VIII concentrate products sold under the Hemofil® M (Baxter, Deerfield, IL), Koate®-DVI (Bayer, Research Tringle Park, NC), Monarc-M™ (American Red Cross, Washington, D.C.), and Monoclate-P® (Aventis, Bridgewater, NJ) brands. With respect to recombinantly prepared Factor VIII concentrates, commercial products are provided under the Helixate® FS (Aventis, Bridgewater, NJ), Kogenate FS® (Bayer, Research Triangle Park, NC), Recombinate® (Baxter, Deerfield, IL), Advate® (Baxter, Deerfield, IL), and ReFacto® (Wyeth/Genetics Institute, Cambridge, MA) brands.

[0008] Generally, recombinant sources of Factor VIII concentrates are favored over blood-derived sources since the latter involves the risk of transmitting viruses and/or other diseases associated with blood donation. While recombinant-based formulations avoid these drawbacks, the processing of recombinant-based products often requires the presence of certain proteins such as albumin, which are inevitably present in the final formulation administered to the patient. Often, patients who receive such formulations develop allergic reactions to these foreign proteins. In any event, both blood-derived and recombinant-based products suffer from the disadvantage of repeated administration.

[0009] PEGylation, or the attachment of a poly(ethylene glycol) derivative to a protein, has been described as a means to reduce immunogenicity as well as a means to prolong a protein's *in vivo* half-life. With respect to Factor VIII, however, previous experiences with forming protein-polymer conjugates has proven to be of little predictive value vis-à-vis polymer coupling to Factor VIII. See U.S. Patent No. 4,970,300.

[0010] Notwithstanding these difficulties, attempts of preparing satisfactory compositions of conjugates of certain polymers to Factor VIII have been described. For example, previously

referenced U.S. Patent No. 4,970,300 describes the PEGylation of Factor VIII using a specific poly(ethylene glycol) derivative having a molecular weight within the range of about 500 to 5,000. In addition, U.S. Patent No. 6,048,720 describes efficient protection against degradation in an *in vitro* environment when four to five monomethoxy poly(ethylene glycol) strands are conjugated to Factor VIII.

[0011] None of these described conjugates, however, has proven to satisfactorily address the problems associated with current Factor VIII-based therapies. For example, conjugates comprised of relatively small polymers (*e.g.*, of about 5,000 Daltons or less) may not suitably provide extended *in vivo* half-life and/or sufficiently reduced immune response. In addition, conjugates having many individual polymers attached to Factor VIII are more likely to have reduced activity as a result of the polymer(s) blocking sites necessary for activity.

[0012] Thus, there remains a need in the art to provide additional conjugates between water-soluble polymers and moieties having Factor VIII activity. The present invention is therefore directed to such conjugates as well as compositions comprising the conjugates and related methods as described herein, which are believed to be new and completely unsuggested by the art.

#### SUMMARY OF THE INVENTION

[0013] Accordingly, it is a primary object of this invention to provide a composition comprising a plurality of conjugates, preferably although not necessarily, each having one to three water-soluble polymers covalently attached to a Factor VIII moiety, wherein each water-soluble polymer preferably has a nominal average molecular weight in the range of greater than 5,000 Daltons to about 100,000 Daltons.

[0014] It is another object of the invention to provide such a conjugate wherein each of the water-soluble polymers is a poly(alkylene oxide).

[0015] It is an additional object of the invention to provide such a conjugate wherein each of the water-soluble polymers is a poly(ethylene glycol).

[0016] It is a further object of the invention to provide a conjugate comprising a plurality of monoPEGylated Factor VIII moiety conjugates.

[0017] It is still a further object of the invention to provide a method for preparing polymer conjugates comprising the steps of contacting one or more activated, water-soluble polymers to a Factor VIII moiety under conditions sufficient to result in a plurality of conjugates, preferably although not necessarily, each having one to three water-soluble polymers covalently attached to a Factor VIII moiety, wherein the water-soluble polymer preferably has a nominal average molecular weight in the range of greater than 5,000 Daltons to about 150,000 Daltons.

[0018] It is an additional object of the invention to provide a method for treating a patient in need of Factor VIII therapy, comprising the step of administering to the patient a composition as described herein, wherein the composition contains a therapeutically effective amount of one or more of the conjugates.

[0019] It is still yet an additional object of the invention to provide a method for preparing a water-soluble polymer-Factor VIII moiety conjugate comprising the step of contacting, under conjugation conditions, a Factor VIII moiety with a polymeric reagent.

[0020] Additional objects, advantages and novel features of the invention will be set forth in the description that follows, and in part, will become apparent to those skilled in the art upon reading the following, or may be learned by practice of the invention.

[0021] In one embodiment then, a composition is provided comprising a plurality of conjugates, preferably although not necessarily, each having one to three-water soluble polymers covalently attached to a Factor VIII moiety, wherein each water-soluble polymer preferably has a nominal average molecular weight in the range of greater than 5,000 Daltons to about 150,000 Daltons. Although any Factor VIII moiety can be used, it is preferred that the compositions comprise Factor VIII *per se* (as described in, for example, U.S. Patent No. 4,757,006), Factor VIIIa (*i.e.*, the activated form of Factor VIII produced when Factor VIII is placed in contact with relatively small amounts of thrombin), Factor VIII:vWF (*i.e.*, Factor VIII bound to von Willebrand Factor), and/or truncated versions of Factor VIII such as B-domain deleted Factor VIII (as described in, for example, U.S. Patent No. 4,868,112).

[0022] The polymer(s) can be any water-soluble polymer and the invention is not limited in this regard. It is preferred, however, that each polymer present in the conjugate is selected from the group consisting of poly(alkylene oxide), poly(vinyl pyrrolidone), poly(vinyl alcohol),

polyoxazoline, poly(acryloylmorpholine), and combinations thereof. It is particularly, preferred, however, that a poly(alkylene oxide) such as a poly(ethylene glycol) derivative is used as the polymer in the present invention.

**[0023]** The conjugates described herein advantageously reduce immunogenicity, a problem encountered by many hemophiliacs treated with exogenous sources of Factor VIII. In addition, the present conjugates require decreased frequency of dosing compared to Factor VIII compositions lacking conjugates. By reducing the frequency of dosing, the conjugates advantageously decrease the number of painful injections hemophiliacs must endure in order to provide sustained levels of an agent having Factor VIII activity.

**[0024]** In another embodiment, a method for preparing a conjugate is provided. The method comprises the step of contacting one or more activated, water-soluble polymers (*i.e.*, a polymeric reagent) preferably having a nominal average molecular weight in the range of greater than 5,000 Daltons to about 150,000 Daltons to a Factor VIII moiety. Activation of the water-soluble polymer can be accomplished under any art-known method so long as the resulting polymer, under the proper conditions of pH, temperature, and so forth, will form a covalent bond such that the Factor VIII moiety is covalently attached to the polymer. Contacting of the one or more activated, water-soluble polymers to the Factor VIII moiety is carried out under those conditions required for the activated, water-soluble polymer to form a covalent attachment at the desired site in the moiety. The method results in a plurality of conjugates, preferably although not necessarily, each having one to three water-soluble polymers covalently attached to the Factor VIII moiety. In some instances, the conjugate can comprise a single polymer attached to two, three, four, five, six, seven, eight, or more Factor VIII moieties. Optionally, the resulting composition can be further processed in order remove undesired species such as, for example, conjugates having an undesired number of polymers. In order to remove such undesired species, purification techniques such as size-exclusion chromatography can be used.

**[0025]** In still another embodiment of the invention, compositions are provided comprising a conjugate of the invention in combination with a pharmaceutically acceptable excipient. The compositions encompass all types of formulations and in particular those that are suited for injection such as powders that can be reconstituted, as well as liquids (*e.g.*, suspensions and solutions).

[0026] In an additional embodiment of the invention, a method of administering the conjugate is provided. The method of administering comprises the step of administering to the patient a composition as described herein, wherein the composition contains a therapeutically effective amount of the conjugate. Typically, the step of administering the conjugate-containing composition is effected by injection (*e.g.*, intramuscular injection, intravenous injection, subcutaneous injection, and so forth).

#### BRIEF DESCRIPTION OF THE FIGURES

[0027] **FIG. 1** is a SEC plot corresponding to the reaction mixture formed upon pegylation of B-Domain deleted Factor VIII with mPEG-SPA, 30K, as described in Example 6.

[0028] **FIG. 2** is a SEC plot corresponding to purified B-Domain Deleted Factor VIII-PEG conjugate prepared by conjugating the protein to mPEG-SPA, 30K, as described in Example 6.

[0029] **FIG. 3** is SEC plot corresponding to the reaction mixture formed upon PEGylation of B-Domain deleted Factor VIII with mPEG-MAL, 20K, as described in Example 7. As can be seen, the yield of monoPEGylated product was approximately 33%.

[0030] **FIG. 4** is a SEC plot corresponding to purified B-Domain Deleted Factor VIII-PEG conjugate prepared by conjugating the protein to mPEG-MAL, 20K, as described in Example 7. The purified conjugate was ~94% PEG monomer.

[0031] **FIG. 5** is a SEC plot corresponding to the reaction mixture formed upon PEGylation of B-Domain Deleted Factor VIII with mPEG-SMB, 30K, as described in Example 8. The yield of monoPEGylated protein (1-mer) was approximately 41%.

#### DETAILED DESCRIPTION OF THE INVENTION

[0032] Before describing the present invention in detail, it is to be understood that this invention is not limited to the particular polymers, synthetic techniques, Factor VIII moieties, and the like, as such may vary.

[0033] It must be noted that, as used in this specification and the intended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a polymer" includes a single polymer as well as two or more of the same or different polymers, reference to a "an optional excipient" refers to a single optional excipient as well as two or more of the same or different optional excipients, and the like.

[0034] In describing and claiming the present invention, the following terminology will be used in accordance with the definitions described below.

[0035] "PEG," "polyethylene glycol" and "poly(ethylene glycol)" as used herein, are interchangeable and meant to encompass any water-soluble poly(ethylene oxide). Typically, PEGs for use in accordance with the invention comprise the following structure "-(OCH<sub>2</sub>CH<sub>2</sub>)<sub>n</sub>-" where (n) is 2 to 4000. As used herein, PEG also includes "-CH<sub>2</sub>CH<sub>2</sub>-O(CH<sub>2</sub>CH<sub>2</sub>O)<sub>n</sub>-CH<sub>2</sub>CH<sub>2</sub>-" and "-(OCH<sub>2</sub>CH<sub>2</sub>)<sub>n</sub>O-," depending upon whether or not the terminal oxygens have been displaced. Throughout the specification and claims, it should be remembered that the term "PEG" includes structures having various terminal or "end capping" groups and so forth. The term "PEG" also means a polymer that contains a majority, that is to say, greater than 50%, of -OCH<sub>2</sub>CH<sub>2</sub>- repeating subunits. With respect to specific forms, the PEG can take any number of a variety of molecular weights, as well as structures or geometries such as "branched," "linear," "forked," "multifunctional," and the like, to be described in greater detail below.

[0036] The terms "end-capped" and "terminally capped" are interchangeably used herein to refer to a terminal or endpoint of a polymer having an end-capping moiety. Typically, although not necessarily, the end-capping moiety comprises a hydroxy or C<sub>1-20</sub> alkoxy group, more preferably a C<sub>1-10</sub> alkoxy group, and still more preferably a C<sub>1-5</sub> alkoxy group. Thus, examples of end-capping moieties include alkoxy (e.g., methoxy, ethoxy and benzyloxy), as well as aryl, heteroaryl, cyclo, heterocyclo, and the like. It must be remembered that the end-capping moiety may include one or more atoms of the terminal monomer in the polymer [e.g., the end-capping moiety "methoxy" in CH<sub>3</sub>(OCH<sub>2</sub>CH<sub>2</sub>)<sub>n</sub>-]. In addition, saturated, unsaturated, substituted and unsubstituted forms of each of the foregoing are envisioned. Moreover, the end-capping group can also be a silane. The end-capping group can also advantageously comprise a detectable label. When the polymer has an end-capping group comprising a

detectable label, the amount or location of the polymer and/or the moiety (*e.g.*, active agent) to which the polymer is coupled can be determined by using a suitable detector. Such labels include, without limitation, fluorescers, chemiluminescers, moieties used in enzyme labeling, colorimetric (*e.g.*, dyes), metal ions, radioactive moieties, and the like. Suitable detectors include photometers, films, spectrometers, and the like. The end-capping group can also advantageously comprise a phospholipid. When the polymer has an end-capping group comprising a phospholipid, unique properties are imparted to the polymer and the resulting conjugate. Exemplary phospholipids include, without limitation, those selected from the class of phospholipids called phosphatidylcholines. Specific phospholipids include, without limitation, those selected from the group consisting of dilaurylphosphatidylcholine, dioleylphosphatidylcholine, dipalmitoylphosphatidylcholine, distearylphosphatidylcholine, behenoylphosphatidylcholine, arachidoylphosphatidylcholine, and lecithin.

[0037] "Non-naturally occurring" with respect to a polymer as described herein, means a polymer that in its entirety is not found in nature. A non-naturally occurring polymer of the invention may, however, contain one or more monomers or segments of monomers that are naturally occurring, so long as the overall polymer structure is not found in nature.

[0038] The term "water soluble" as in a "water-soluble polymer" polymer is any polymer that is soluble in water at room temperature. Typically, a water-soluble polymer will transmit at least about 75%, more preferably at least about 95%, of light transmitted by the same solution after filtering. On a weight basis, a water-soluble polymer will preferably be at least about 35% (by weight) soluble in water, more preferably at least about 50% (by weight) soluble in water, still more preferably about 70% (by weight) soluble in water, and still more preferably about 85% (by weight) soluble in water. It is most preferred, however, that the water-soluble polymer is about 95% (by weight) soluble in water or completely soluble in water.

[0039] "Nominal average molecular weight" in the context of a water-soluble, non-naturally occurring polymer such as PEG, refers to the mass average molecular weight of the polymer, typically determined by size-exclusion chromatography, MALDI (matrix assisted laser desorption/ionization), light scattering techniques, or intrinsic velocity determination in 1,2,4-trichlorobenzene. The polymers are typically polydisperse, possessing low polydispersity values of preferably less than about 1.2, more preferably less than about 1.15, still more

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preferably less than about 1.10, yet still more preferably less than about 1.05, and most preferably less than about 1.03.

[0040] The term "active" or "activated" when used in conjunction with a particular functional group, refers to a reactive functional group that reacts readily with an electrophile or a nucleophile on another molecule. This is in contrast to those groups that require strong catalysts or highly impractical reaction conditions in order to react (*i.e.*, a "non-reactive" or "inert" group).

[0041] As used herein, the term "functional group" or any synonym thereof is meant to encompass protected forms thereof as well as unprotected forms.

[0042] The terms "linkage" or "linker" are used herein to refer to an atom or a collection of atoms optionally used to link interconnecting moieties such as a terminus of a polymer segment and a Factor VIII moiety or an electrophile or nucleophile of a Factor VIII moiety. The linker of the invention may be hydrolytically stable or may include a physiologically hydrolyzable or enzymatically degradable linkage.

[0043] "Alkyl" refers to a hydrocarbon chain, typically ranging from about 1 to 15 atoms in length. Such hydrocarbon chains are preferably but not necessarily saturated and may be branched or straight chain, although typically straight chain is preferred. Exemplary alkyl groups include methyl, ethyl, propyl, butyl, pentyl, 1-methylbutyl, 1-ethylpropyl, 3-methylpentyl, and the like. As used herein, "alkyl" includes cycloalkyl as well as cycloalkylene-containing alkyl.

[0044] "Lower alkyl" refers to an alkyl group containing from 1 to 6 carbon atoms, and may be straight chain or branched, as exemplified by methyl, ethyl, *n*-butyl, *i*-butyl, and *t*-butyl.

[0045] "Cycloalkyl" refers to a saturated or unsaturated cyclic hydrocarbon chain, including bridged, fused, or spiro cyclic compounds, preferably made up of 3 to about 12 carbon atoms, more preferably 3 to about 8 carbon atoms. "Cycloalkylene" refers to a cycloalkyl group that is inserted into an alkyl chain by bonding of the chain at any two carbons in the cyclic ring system.

[0046] "Alkoxy" refers to an -O-R group, wherein R is alkyl or substituted alkyl, preferably C<sub>1-6</sub> alkyl (*e.g.*, methoxy, ethoxy, propyloxy, and so forth).

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[0047] The term "substituted" as in, for example, "substituted alkyl," refers to a moiety (*e.g.*, an alkyl group) substituted with one or more noninterfering substituents, such as, but not limited to: alkyl, C<sub>3</sub>-8 cycloalkyl, *e.g.*, cyclopropyl, cyclobutyl, and the like; halo, *e.g.*, fluoro, chloro, bromo, and iodo; cyano; alkoxy, lower phenyl; substituted phenyl; and the like. "Substituted aryl" is aryl having one or more noninterfering groups as a substituent. For substitutions on a phenyl ring, the substituents may be in any orientation (*i.e.*, ortho, meta, or para).

[0048] "Noninterfering substituents" are those groups that, when present in a molecule, are typically nonreactive with other functional groups contained within the molecule.

[0049] "Aryl" means one or more aromatic rings, each of 5 or 6 core carbon atoms. Aryl includes multiple aryl rings that may be fused, as in naphthyl or unfused, as in biphenyl. Aryl rings may also be fused or unfused with one or more cyclic hydrocarbon, heteroaryl, or heterocyclic rings. As used herein, "aryl" includes heteroaryl.

[0050] "Heteroaryl" is an aryl group containing from one to four heteroatoms, preferably sulfur, oxygen, or nitrogen, or a combination thereof. Heteroaryl rings may also be fused with one or more cyclic hydrocarbon, heterocyclic, aryl, or heteroaryl rings.

[0051] "Heterocycle" or "heterocyclic" means one or more rings of 5-12 atoms, preferably 5-7 atoms, with or without unsaturation or aromatic character and having at least one ring atom that is not a carbon. Preferred heteroatoms include sulfur, oxygen, and nitrogen.

[0052] "Substituted heteroaryl" is heteroaryl having one or more noninterfering groups as substituents.

[0053] "Substituted heterocycle" is a heterocycle having one or more side chains formed from noninterfering substituents.

[0054] "Electrophile" and "electrophilic group" refer to an ion or atom or collection of atoms, that may be ionic, having an electrophilic center, *i.e.*, a center that is electron seeking, capable of reacting with a nucleophile.

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[0055] "Nucleophile" and "nucelophilic group" refers to an ion or atom or collection of atoms that may be ionic having a nucleophilic center, *i.e.*, a center that is seeking an electrophilic center or with an electrophile.

[0056] A "physiologically cleavable" or "hydrolysable" or "degradable" bond is a bond that reacts with water (*i.e.*, is hydrolyzed) under physiological conditions. Preferred are bonds that have a hydrolysis half-life at pH 8, 25 °C of less than about 30 minutes. The tendency of a bond to hydrolyze in water will depend not only on the general type of linkage connecting two central atoms but also on the substituents attached to these central atoms. Appropriate hydrolytically unstable or weak linkages include but are not limited to carboxylate ester, phosphate ester, anhydrides, acetals, ketals, acyloxyalkyl ether, imines, orthoesters, peptides and oligonucleotides.

[0057] An "enzymatically degradable linkage" means a linkage that is subject to degradation by one or more enzymes.

[0058] A "hydrolytically stable" linkage or bond refers to a chemical bond, typically a covalent bond, that is substantially stable in water, that is to say, does not undergo hydrolysis under physiological conditions to any appreciable extent over an extended period of time. Examples of hydrolytically stable linkages include, but are not limited to, the following: carbon-carbon bonds (*e.g.*, in aliphatic chains), ethers, amides, urethanes, and the like. Generally, a hydrolytically stable linkage is one that exhibits a rate of hydrolysis of less than about 1-2% per day under physiological conditions. Hydrolysis rates of representative chemical bonds can be found in most standard chemistry textbooks.

[0059] "Pharmaceutically acceptable excipient or carrier" refers to an excipient that may optionally be included in the compositions of the invention and that causes no significant adverse toxicological effects to the patient. "Pharmacologically effective amount," "physiologically effective amount," and "therapeutically effective amount" are used interchangeably herein to mean the amount of a polymer-Factor VIII moiety conjugate that is needed to provide a desired level of the conjugate (or corresponding unconjugated Factor VIII moiety) in the bloodstream or in the target tissue. The precise amount will depend upon numerous factors, *e.g.*, the particular Factor VIII moiety, the components and physical characteristics of the therapeutic composition, intended

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patient population, individual patient considerations, and the like, and can readily be determined by one skilled in the art, based upon the information provided herein.

[0060] "Multi-functional" means a polymer having 3 or more functional groups contained therein, where the functional groups may be the same or different. Multi-functional polymeric reagents of the invention will typically contain from about 3-100 functional groups, or from 3-50 functional groups, or from 3-25 functional groups, or from 3-15 functional groups, or from 3 to 10 functional groups, or will contain 3, 4, 5, 6, 7, 8, 9 or 10 functional groups within the polymer backbone.

[0061] The term "Factor VIII moiety," as used herein, refers to a moiety having Factor VIII activity. The Factor VIII moiety will also have at least one electrophilic group or nucleophilic group suitable for reaction with a polymeric reagent. Typically, although not necessarily, the Factor VIII moiety is a protein. In addition, the term "Factor VIII moiety" encompasses both the Factor VIII moiety prior to conjugation as well as the Factor VIII moiety residue following conjugation. As will be explained in further detail below, one of ordinary skill in the art can determine whether any given moiety has Factor VIII activity.

[0062] The term "patient," refers to a living organism suffering from or prone to a condition that can be prevented or treated by administration of an active agent (*e.g.*, conjugate), and includes both humans and animals.

[0063] "Optional" or "optionally" means that the subsequently described circumstance may or may not occur, so that the description includes instances where the circumstance occurs and instances where it does not.

[0064] Amino acid residues in peptides are abbreviated as follows: Phenylalanine is Phe or F; Leucine is Leu or L; Isoleucine is Ile or I; Methionine is Met or M; Valine is Val or V; Serine is Ser or S; Proline is Pro or P; Threonine is Thr or T; Alanine is Ala or A; Tyrosine is Tyr or Y; Histidine is His or H; Glutamine is Gln or Q; Asparagine is Asn or N; Lysine is Lys or K; Aspartic Acid is Asp or D; Glutamic Acid is Glu or E; Cysteine is Cys or C; Tryptophan is Trp or W; Arginine is Arg or R; and Glycine is Gly or G.

[0065] Turning to a first embodiment of the invention then, a composition is provided comprising a plurality of conjugates, preferably although not necessarily, each having one to

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three water-soluble polymers covalently attached to a Factor VIII moiety, wherein each of the water-soluble polymers preferably has a nominal average molecular weight in the range of greater than 5,000 Daltons to about 150,000 Daltons.

[0066] Native Factor VIII is a 2,351 amino acid, single chain glycoprotein that is structurally organized as A1-A2-B-A3-C1-C2. The expressed 2,351 amino acid sequence is provided as SEQ. ID. NO: 1. When the expressed polypeptide is translocated into the lumen of the endoplasmic reticulum, however, a 19-amino acid signal sequence is cleaved, resulting in a second sequence. This second sequence, herein provided as SEQ. ID. NO: 2, lacks the leading 19 amino acids is conventionally used by researchers to assign a numeric location (*e.g.*, Arg<sup>372</sup>) to a given amino acid residue of Factor VIII. Thus, unless specifically noted, all assignments of a numeric location of an amino acid residue as provided herein are based on SEQ. ID. NO: 2.

[0067] In the presence of relatively small amounts of thrombin, Factor VIII is cleaved by thrombin at Arg<sup>372</sup>, Arg<sup>740</sup>, and Arg<sup>1689</sup> to produce Factor VIIIa. Factor VIIIa is a heterotrimer comprised of the A1 subunit bound (via a copper ion) to the thrombin-cleaved light chain A3-C1-C2 and the free A2 subunit bound via ionic interactions to A1. It will be appreciated that a Factor VIII moiety is not limited to merely "active" forms of Factor VIII (*e.g.*, Factor VIIIa) and that the term "Factor VIII moiety" encompasses "precursor" forms as well as other substances that having a similar procoagulant effect .

[0068] For any given moiety, it is possible to determine whether that moiety has Factor VIII activity. For example, several animal lines have been intentionally bred with the genetic mutation for hemophilia such that an animal produced from such a line has very low and insufficient levels of Factor VIII. Such lines are available from a variety of sources such as, without limitation, the Division of Laboratories and Research, New York Department of Public Health, Albany, NY and the Department of Pathology, University of North Carolina, Chapel Hill, NC. Both of these sources, for example, provide canines suffering from canine hemophilia A. In order to test the Factor VIII activity of any given moiety in question, the moiety is injected into the diseased animal, a small cut made and bleeding time compared to a healthy control. Another method useful for determining Factor VIII activity is to determine cofactor and procoagulant activity. See, for example, Mertens *et al.* (1993) *Brit. J. Haematol.* 85:133-42. Other methods known to those of ordinary skill in the art can also be used to determine whether a given moiety

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has Factor VIII activity. Such methods are useful for determining the Factor VIII activity of both the moiety itself (and therefore can be used as a "Factor VIII moiety) as well as the corresponding polymer-moiety conjugate.

[0069] Nonlimiting examples of Factor VIII moieties include the following: Factor VIII; Factor VIIIa; Factor VIII:C; Factor VIII:vWF; B-domain deleted Factor VIII (and other truncated versions of Factor VIII); hybrid proteins, such as those described in U.S. Patent No. 6,158,888; glycosylated proteins having Factor VIII activity, such as those described in U.S. Patent Application Publication No. US2003/0077752; and peptide mimetics having Factor VIII activity. Preferred truncated Factor VIII versions (encompassed by the term "B-domain deleted Factor VIII) corresponds to a protein having the amino acid sequence of human Factor VIII (SEQ. ID. NO. 1) having a deletion corresponding to at least 581 amino acids within the region between Arg<sup>759</sup> and Ser<sup>1709</sup>, more preferably wherein the deletion corresponds to one of the region between Pro<sup>1000</sup> and Asp<sup>1582</sup>, the region between Thr<sup>778</sup> and Pro<sup>1659</sup>, and the region between Thr<sup>778</sup> and Glu<sup>1694</sup>. Biologically active fragments, deletion variants, substitution variants or addition variants of any of the foregoing that maintain at least some degree of Factor VIII activity can also serve as a Factor VIII moiety.

[0070] The moiety having Factor VIII activity can advantageously be modified to include one or more amino acid residues such as, for example, lysine, cysteine and/or arginine, in order to provide facile attachment of the polymer to an atom within the side chain of the amino acid. Techniques for adding amino acid residues are well known to those of ordinary skill in the art. Reference is made to J. March, Advanced Organic Chemistry: Reactions Mechanisms and Structure, 4th Ed. (New York: Wiley-Interscience, 1992).

[0071] The Factor VIII moiety can be obtained from blood-derived sources. For example, Factor VIII can be fractionated from human plasma using precipitation and centrifugation techniques known to those of ordinary skill in the art. See, for example, Wickerhauser (1976) *Transfusion* 16(4):345-350 and Slichter *et al.* (1976) *Transfusion* 16(6):616-626. Factor VIII can also be isolated from human granulocytes. See Szmitskoski *et al.* (1977) *Haematologia (Budap.)* 11(1-2):177-187.

[0072] In addition, the Factor VIII moiety can also be obtained from recombinant methods. Briefly, recombinant methods involve constructing the nucleic acid encoding the desired polypeptide or fragment, cloning the nucleic acid into an expression vector, transforming a host cell (*e.g.*, bacteria, yeast, or mammalian cell such as Chinese hamster ovary cell or baby hamster kidney cell), and expressing the nucleic acid to produce the desired polypeptide or fragment. Methods for producing and expressing recombinant polypeptides *in vitro* and in prokaryotic and eukaryotic host cells are known to those of ordinary skill in the art. See, for example, U.S. Patent No. 4,868,122.

[0073] To facilitate identification and purification of the recombinant polypeptide, nucleic acid sequences that encode for an epitope tag or other affinity binding sequence can be inserted or added in-frame with the coding sequence, thereby producing a fusion protein comprised of the desired polypeptide and a polypeptide suited for binding. Fusion proteins can be identified and purified by first running a mixture containing the fusion protein through an affinity column bearing binding moieties (*e.g.*, antibodies) directed against the epitope tag or other binding sequence in the fusion proteins, thereby binding the fusion protein within the column. Thereafter, the fusion protein can be recovered by washing the column with the appropriate solution (*e.g.*, acid) to release the bound fusion protein. The recombinant polypeptide can also be identified and purified by lysing the host cells, separating the polypeptide, *e.g.*, by size exclusion chromatography, and collecting the polypeptide. These and other methods for identifying and purifying recombinant polypeptides are known to those of ordinary skill in the art.

[0074] The compositions of the invention can comprise a plurality of conjugates, each conjugate comprised of the same Factor VIII moiety (*i.e.*, within the entire composition, only one type of Factor VIII moiety is found). In addition, the composition can comprise a plurality of conjugates wherein any given conjugate is comprised of a moiety selected from the group consisting of two or more different Factor VIII moieties (*i.e.*, within the entire composition, two or more different Factor VIII moieties are found). Optimally, however, substantially all of the plurality of conjugates in the composition (*e.g.*, 85% or more of the plurality of conjugates in the composition) are each comprised of the same Factor VIII moiety.

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[0075] Moreover, it is preferred that the composition containing the conjugates is free or substantially free from albumin. It is also preferred that the composition is free or substantially free proteins that do not have Factor VIII activity. Thus, it is preferred that the composition is 85%, more preferably 95%, and most preferably 99% free from albumin. Additionally, it is preferred that the composition is 85%, more preferably 95%, and most preferably 99% free from any protein that does not have Factor VIII activity.

[0076] As previously discussed, each conjugate comprises a Factor VIII moiety attached to a water-soluble polymer. With respect to the water-soluble polymer, the water-soluble polymer is nonpeptidic, nontoxic, non-naturally occurring and biocompatible. With respect to biocompatibility, a substance is considered biocompatible if the beneficial effects associated with use of the substance alone or with another substance (*e.g.*, active agent such a Factor VIII moiety) in connection with living tissues (*e.g.*, administration to a patient) outweighs any deleterious effects as evaluated by a clinician, *e.g.*, a physician. With respect to non-immunogenicity, a substance is considered nonimmunogenic if the intended use of the substance *in vivo* does not produce an undesired immune response (*e.g.*, the formation of antibodies) or, if an immune response is produced, that such a response is not deemed clinically significant or important as evaluated by a clinician. It is particularly preferred that the water-soluble polymer is biocompatible and nonimmunogenic.

[0077] Further the polymer is typically characterized as having from 2 to about 300 termini. Examples of such polymers include, but are not limited to, poly(alkylene glycols) such as polyethylene glycol (PEG), poly(propylene glycol) ("PPG"), copolymers of ethylene glycol and propylene glycol and the like, poly(oxyethylated polyol), poly(olefinic alcohol), poly(vinylpyrrolidone), poly(hydroxyalkylmethacrylamide), poly(hydroxyalkylmethacrylate), poly(saccharides), poly( $\alpha$ -hydroxy acid), poly(vinyl alcohol), polyphosphazene, polyoxazoline, poly(N-acryloylmorpholine), and combinations of any of the foregoing.

[0078] The polymer is not limited in a particular structure and can be linear (*e.g.*, alkoxy PEG or bifunctional PEG), branched or multi-armed (*e.g.*, forked PEG or PEG attached to a polyol core), dendritic, or with degradable linkages. Moreover, the internal structure of the polymer can be organized in any of number of different patterns and can be selected from the

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group consisting of homopolymer, alternating copolymer, random copolymer, block copolymer, alternating tripolymer, random tripolymer, and block tripolymer.

[0079] Typically, PEG and other water-soluble polymers are activated with a suitable activating group appropriate for coupling to a desired site on the Factor VIII moiety. An activated polymeric reagent will possess a reactive group for reaction with the Factor VIII moiety. Representative polymeric reagents and methods for conjugating these polymers to an active moiety are known in the art and further described in Zalipsky, S., *et al.*, "Use of Functionalized Poly(Ethylene Glycols) for Modification of Polypeptides" in Polyethylene Glycol Chemistry: Biotechnical and Biomedical Applications, J. M. Harris, Plenus Press, New York (1992), and in Zalipsky (1995) *Advanced Drug Reviews* 16:157-182.

[0080] Typically, the nominal average molecular weight of any given polymer in the conjugate is from about 100 Daltons to about 150,000 Daltons. Exemplary ranges, however, include nominal average molecular weights in the range of greater than 5,000 Daltons to about 100,000 Daltons, in the range of from about 6,000 Daltons to about 90,000 Daltons, in the range of from about 10,000 Daltons to about 85,000 Daltons, in the range of from about 20,000 Daltons to about 85,000 Daltons, and in the range of from about 53,000 Daltons to about 85,000 Daltons. For any given water-soluble polymer, PEGs having these molecular weight ranges are preferred.

[0081] Exemplary nominal average molecular weights for the water-soluble polymer segment include about 100 Daltons, about 200 Daltons, about 300 Daltons, about 400 Daltons, about 500 Daltons, about 600 Daltons, about 700 Daltons, about 750 Daltons, about 800 Daltons, about 900 Daltons, about 1,000 Daltons, about 2,000 Daltons, about 2,200 Daltons, about 2,500 Daltons, about 3,000 Daltons, about 4,000 Daltons, about 4,400 Daltons, about 5,000 Daltons, about 6,000 Daltons, about 7,000 Daltons, about 7,500 Daltons, about 8,000 Daltons, about 9,000 Daltons, about 10,000 Daltons, about 11,000 Daltons, about 12,000 Daltons, about 13,000 Daltons, about 14,000 Daltons, about 15,000 Daltons, about 20,000 Daltons, about 22,500 Daltons, about 25,000 Daltons, about 30,000 Daltons, about 40,000 Daltons, about 50,000 Daltons, about 60,000 Daltons, and about 75,000 Daltons.

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[0082] When used as the polymer, PEGs will typically comprise a number of (OCH<sub>2</sub>CH<sub>2</sub>) monomers. As used throughout the description, the number of repeating units is identified by the subscript "n" in "(OCH<sub>2</sub>CH<sub>2</sub>)<sub>n</sub>." Thus, the value of (n) typically falls within one or more of the following ranges: from 2 to about 2,300, from about 100 to about 2,300, from about 135 to about 2,000, from about 230 to about 1,900, from about 450 to about 1,900, and from about 1,200 to about 1,900. For any given polymer in which the molecular weight is known, it is possible to determine the number of repeating units (i.e., "n") by dividing the total molecular weight of the polymer by the molecular weight of the repeating unit.

[0083] One particularly preferred polymer for use in the invention is an end-capped polymer, that is, a polymer having at least one terminus capped with a relatively inert group, such as a lower C<sub>1-6</sub> alkoxy group. When the polymer is PEG, for example, it is preferred to use a methoxy-PEG (commonly referred to as mPEG), which is a linear form of PEG wherein one terminus of the polymer is a methoxy (-OCH<sub>3</sub>) group, while the other terminus is a hydroxyl or other functional group that can be optionally chemically modified.

[0084] In one form useful in the present invention, free or unbound PEG is a linear polymer terminated at each end with hydroxyl groups:



wherein (m') typically ranges from zero to about 4,000.

[0085] The above polymer, alpha-, omega-dihydroxylpoly(ethylene glycol), can be represented in brief form as HO-PEG-OH where it is understood that the -PEG- symbol can represent the following structural unit:



wherein (m') is as defined as above.

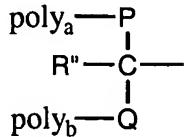
[0086] Another type of PEG useful in the present invention is methoxy-PEG-OH, or mPEG in brief, in which one terminus is the relatively inert methoxy group, while the other terminus is a hydroxyl group. The structure of mPEG is given below.



wherein (m') is as described above.

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[0087] Multi-armed or branched PEG molecules, such as those described in U.S. Patent No. 5,932,462, can also be used as the PEG polymer. For example, PEG can have the structure:



[0088] wherein:

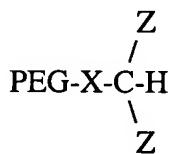
[0089] poly<sub>a</sub> and poly<sub>b</sub> are PEG backbones (either the same or different), such as methoxy poly(ethylene glycol);

[0090] R" is a nonreactive moiety, such as H, methyl or a PEG backbone; and

[0091] P and Q are nonreactive linkages. In a preferred embodiment, the branched PEG polymer is methoxy poly(ethylene glycol) disubstituted lysine. Depending on the specific Factor VIII moiety used, the reactive ester functional group of the disubstituted lysine may be further modified to form a functional group suitable for reaction with the target group within the Factor VIII moiety.

[0092] These polymers may be linear, or may be in any of the above-described forms.

[0093] In addition, the PEG can comprise a forked PEG. An example of a forked PEG is represented by the following structure:



[0094] wherein: X is a spacer moiety of one or more atoms and each Z is an activated terminal group linked to CH by a chain of atoms of defined length. International Application No. PCT/US99/05333, discloses various forked PEG structures capable of use in the present invention. The chain of atoms linking the Z functional groups to the branching carbon atom serve as a tethering group and may comprise, for example, alkyl chains, ether chains, ester chains, amide chains and combinations thereof.

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[0095] The PEG polymer may comprise a pendant PEG molecule having reactive groups, such as carboxyl, covalently attached along the length of the PEG rather than at the end of the PEG chain. The pendant reactive groups can be attached to the PEG directly or through a spacer moiety, such as an alkylene group.

[0096] In addition to the above-described forms of PEG, the polymer can also be prepared with one or more weak or degradable linkages in the polymer, including any of the above described polymers. For example, PEG can be prepared with ester linkages in the polymer that are subject to hydrolysis. As shown below, this hydrolysis results in cleavage of the polymer into fragments of lower molecular weight:



[0097] Other hydrolytically degradable linkages, useful as a degradable linkage within a polymer backbone, include: carbonate linkages; imine linkages resulting, for example, from reaction of an amine and an aldehyde (see, e.g., Ouchi *et al.* (1997) *Polymer Preprints* 38(1):582-3); phosphate ester linkages formed, for example, by reacting an alcohol with a phosphate group; hydrazone linkages which are typically formed by reaction of a hydrazide and an aldehyde; acetal linkages that are typically formed by reaction between an aldehyde and an alcohol; orthoester linkages that are, for example, formed by reaction between a formate and an alcohol; amide linkages formed by an amine group, *e.g.*, at an end of a polymer such as PEG, and a carboxyl group of another PEG chain; urethane linkages formed from reaction of, *e.g.*, a PEG with a terminal isocyanate group and a PEG alcohol; peptide linkages formed by an amine group, *e.g.*, at an end of a polymer such as PEG, and a carboxyl group of a peptide; and oligonucleotide linkages formed by, for example, a phosphoramidite group, *e.g.*, at the end of a polymer, and a 5' hydroxyl group of an oligonucleotide.

[0098] Such optional features of the polymer conjugate, *i.e.*, the introduction of one or more degradable linkages into the polymer chain, may provide for additional control over the final desired pharmacological properties of the conjugate upon administration. For example, a large and relatively inert conjugate (*i.e.*, having one or more high molecular weight PEG chains attached thereto, for example, one or more PEG chains having a molecular weight greater than about 10,000, wherein the conjugate possesses essentially no bioactivity) may be administered,

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which is hydrolyzed to generate a bioactive conjugate possessing a portion of the original PEG chain. In this way, the properties of the conjugate can be more effectively tailored to balance the bioactivity of the conjugate over time.

[0099] Those of ordinary skill in the art will recognize that the foregoing discussion concerning substantially water-soluble polymer segments is by no means exhaustive and is merely illustrative, and that all polymeric materials having the qualities described above are contemplated. As used herein, the term "polymeric reagent" generally refers to an entire molecule, which can comprise a water-soluble polymer segment and a functional group.

[0100] As described above, a conjugate of the invention comprises a water-soluble polymer covalently attached to a Factor VIII moiety. Typically, for any given conjugate, there will be one to three water-soluble polymers covalently attached to one or more moieties having Factor VIII activity. In some instances, however, the conjugate may have 1, 2, 3, 4, 5, 6, 7, 8 or more water-soluble polymers individually attached to a Factor VIII moiety.

[0101] The particular linkage within the moiety having Factor VIII activity and the polymer depends on a number of factors. Such factors include, for example, the particular linkage chemistry employed, the particular moiety having Factor VIII activity, the available functional groups within the moiety having Factor VIII activity (either for attachment to a polymer or conversion to a suitable attachment site), the possible presence of additional reactive functional groups within the moiety having Factor VIII activity, and the like.

[0102] The conjugates of the invention can be, although not necessarily, prodrugs, meaning that the linkage between the polymer and the Factor VIII moiety is hydrolytically degradable to allow release of the parent moiety. Exemplary degradable linkages include carboxylate ester, phosphate ester, thiolester, anhydrides, acetals, ketals, acyloxyalkyl ether, imines, orthoesters, peptides and oligonucleotides. Such linkages can be readily prepared by appropriate modification of either the Factor VIII moiety (*e.g.*, the carboxyl group C terminus of the protein or a side chain hydroxyl group of an amino acid such as serine or threonine contained within the protein) and/or the polymeric reagent using coupling methods commonly employed in the art. Most preferred, however, are hydrolyzable linkages that are readily formed by reaction

of a suitably activated polymer with a non-modified functional group contained within the moiety having Factor VIII activity.

[0103] Alternatively, a hydrolytically stable linkage, such as an amide, urethane (also known as carbamate), amine, thioether (also known as sulfide), or urea (also known as carbamide) linkage can also be employed as the linkage for coupling the Factor VIII moiety. In some cases, however, it is preferred that the linkage is not a carbamate linkage and not a carbamide linkage, and furthermore, that no linkage is formed based on the reaction of a polymer derivative bearing an isocyanate or isothiocyanate species to a Factor VIII moiety. Again, a preferred hydrolytically stable linkage is an amide. An amide can be readily prepared by reaction of a carboxyl group contained within the Factor VIII moiety (*e.g.*, the terminal carboxyl of a peptidic moiety having Factor VIII activity) with an amino-terminated polymer.

[0104] The conjugates (as opposed to an unconjugated Factor VIII moiety) may or may not possess a measurable degree of Factor VIII activity. That is to say, a polymer conjugate in accordance with the invention will possesses anywhere from about 0.1% to about 100% or more of the bioactivity of the unmodified parent Factor VIII moiety. Preferably, compounds possessing little or no Factor VIII activity typically contain a hydrolyzable linkage connecting the polymer to the moiety, so that regardless of the lack of activity in the conjugate, the active parent molecule (or a derivative thereof) is released upon aqueous-induced cleavage of the hydrolyzable linkage. Such activity may be determined using a suitable *in-vivo* or *in-vitro* model, depending upon the known activity of the particular moiety having Factor VIII activity employed.

[0105] For conjugates possessing a hydrolytically stable linkage that couples the moiety having Factor VIII activity to the polymer, the conjugate will typically possess a measurable degree of specific activity. For instance, such polymer conjugates are typically characterized as having an activity of at least about 2%, 5%, 10%, 15%, 25%, 30%, 40%, 50%, 60%, 80%, 85%, 90%, 95% 97%, 100%, or more relative to that of the unmodified parent moiety having Factor VIII activity, when measured in a suitable model, such as those well known in the art. Preferably, compounds having a hydrolytically stable linkage (*e.g.*, an amide linkage) will possess at least some degree of the bioactivity of the unmodified parent moiety having Factor VIII activity.

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[0106] Exemplary polymer conjugates in accordance with the invention will now be described wherein the moiety having Factor VIII activity is a protein. Typically, such a protein is expected to share (at least in part) a similar amino acid sequence as native Factor VIII. Thus, while reference will be made to specific locations or atoms within the native Factor VIII protein, such a reference is for convenience only and one having ordinary skill in the art will be able to readily determine the corresponding location or atom in other moieties having Factor VIII activity. In particular, the description provided herein for native Factor VIII is applicable to Factor VIIIa, Factor VIII:vWF, and B-domain deleted Factor VIII versions, as well as fragments, deletion variants, substitution variants or addition variants of any of the foregoing.

[0107] Amino groups on Factor VIII moieties provide a point of attachment between the Factor VIII moiety and the water-soluble polymer. Native Factor VIII comprises 158 amine-containing lysine residues (6.8 weight percent of the entire protein) and one amino terminus. With respect to Factor VIIIa, there are 78 lysine residues (5.5 weight percent of the entire protein) and two amino termini (resulting from the cleavage of Factor VIII). Consequently, notwithstanding secondary and tertiary structural considerations, both Factor VIII and Factor VIIIa (as well as any peptidic Factor VIII moiety, *e.g.*, B-domain deleted Factor VIII) have several amines available for participation in conjugating reactions.

[0108] There are a number of examples of suitable water-soluble polymeric reagents useful for forming covalent linkages with available amines of a Factor VIII moiety. Specific examples, along with the corresponding conjugate, are provided in Table 1, below. In the table, the variable (n) represents the number of repeating monomeric units and "-NH-F8" represents the Factor VIII moiety following conjugation to the water-soluble polymer. While each polymeric portion presented in Table 1 terminates in a "CH<sub>3</sub>" group, other groups (such as H and benzyl) can be substituted therefor.

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Table 1

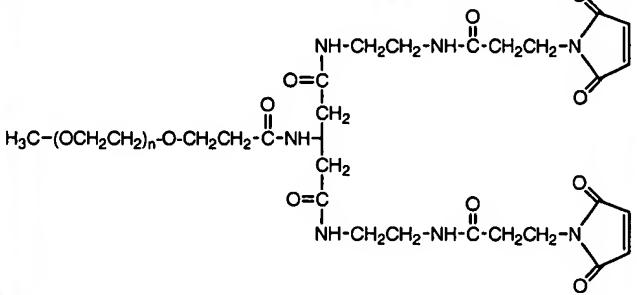
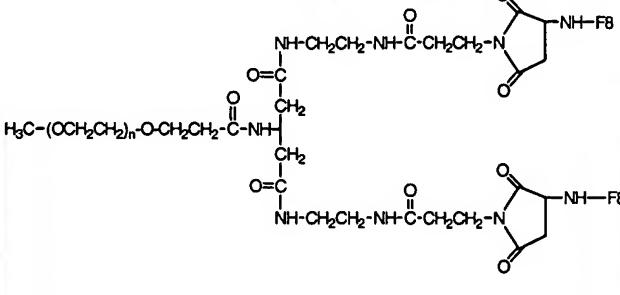
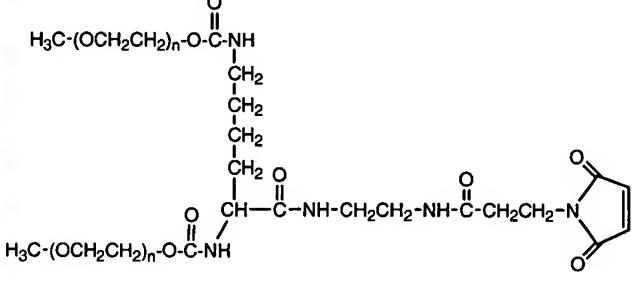
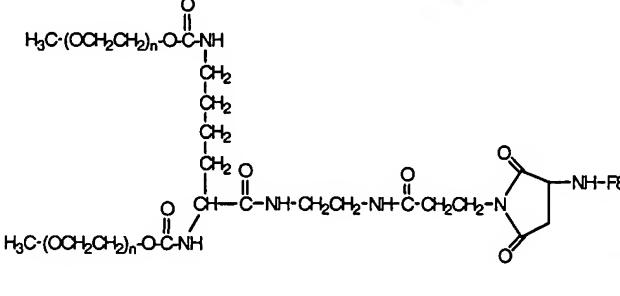
Amine-Specific Polymeric Reagents and the Factor VIII Moiety Conjugate Formed Therefrom

| Polymeric Reagent  | Corresponding Conjugate  |
|--|--|
| $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\text{C}-\text{O}-\text{N}$<br>mPEG-Succinimidyl Propionate  | $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}\text{C}-\text{NH}-\text{F8}$<br>Amide Linkage  |
| $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\text{C}-\text{O}-\text{N}$<br>Homobifunctional PEG-Succinimidyl Propionate                                    | $\text{F8}-\text{NH}-\overset{\text{O}}{\parallel}\text{C}-\text{CH}_2\text{CH}_2-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}\text{C}-\text{NH}-\text{F8}$<br>Amide Linkages         |
| $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2\text{CH}_2-\text{C}-\text{O}-\text{N}$<br>mPEG-Succinimidyl Butanoate  | $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}\text{C}-\text{NH}-\text{F8}$<br>Amide Linkage   |
| $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{C}-\text{O}-\text{N}$<br>mPEG-Benzotriazole Carbonate   | $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\overset{\text{O}}{\parallel}\text{C}-\text{NH}-\text{F8}$<br>Carbamate Linkage   |
| $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{NH}-\text{C}$<br>mPEG-Succinimidyl Derivative  | $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{NH}-\text{C}$<br>Carbamate Linkage   |
| $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{C}-\text{NH}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{C}-\text{O}-\text{N}$<br>Branched mPEG2-N-Hydroxysuccinimide | $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\overset{\text{O}}{\parallel}\text{C}-\text{NH}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\overset{\text{O}}{\parallel}\text{C}-\text{O}-\text{N}$<br>Amide Linkage |

| Polymeric Reagent  | Corresponding Conjugate  |
|--|--|
| $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\overset{\text{O}}{\parallel}\text{C}-\text{O}-\overset{\text{CH}_3}{\text{CH}}\text{CH}_2-\overset{\text{O}}{\parallel}\text{C}-\text{O}-\text{N}$<br><p>mPEG-Succinimidyl Derivative</p> | $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\overset{\text{O}}{\parallel}\text{C}-\text{O}-\overset{\text{CH}_3}{\text{CH}}\text{CH}_2-\overset{\text{O}}{\parallel}\text{C}-\text{NH}-\text{F8}$<br><p>Amide Linkage</p>  |
| $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2\overset{\text{O}}{\parallel}\text{C}-\text{CH}-\overset{\text{CH}_3}{\text{C}}-\text{O}-\text{N}$<br><p>mPEG-Succinimidyl Derivative</p>                                       | $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2\overset{\text{O}}{\parallel}\text{C}-\text{CH}-\overset{\text{CH}_3}{\text{C}}-\text{NH}-\text{F8}$<br><p>Amide Linkage</p>  |
| $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2\overset{\text{O}}{\parallel}\text{C}-\text{S}-\text{C}_6\text{H}_4-\text{N}$<br>   | $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2\overset{\text{O}}{\parallel}\text{C}-\text{NH}-\text{F8}$<br><p>Amide Linkage</p>  |
| $\text{HC}-\overset{\text{O}}{\parallel}\text{CH}_2\text{CH}_2-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}\text{CH}$<br><p>Homobifunctional PEG Propionaldehyde</p>  | $\text{F8}-\text{NH}-\text{CH}_2\text{CH}_2\text{CH}_2-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2\text{CH}_2-\text{NH}-\text{F8}$<br><p>Secondary Amine Linkages</p>  |
| $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\overset{\text{O}}{\parallel}\text{C}-\text{CH}$<br><p>mPEG Propionaldehyde</p>  | $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}\text{CH}-\text{NH}-\text{F8}$<br><p>Secondary Amine Linkage</p>   |
| $\text{HCCH}_2\text{CH}_2\text{CH}_2-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2\text{CH}_2\overset{\text{O}}{\parallel}\text{CH}$<br><p>Homobifunctional PEG Butyraldehyde</p>  | $\text{F8}-\text{NH}-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}\text{CH}-\text{NH}-\text{F8}$<br><p>Secondary Amine Linkage</p>   |
| $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2\text{CH}_2\overset{\text{O}}{\parallel}\text{CH}$<br><p>mPEG Butyraldehyde</p>   | $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}\text{CH}-\text{NH}-\text{F8}$<br><p>Secondary Amine Linkage</p>  |
| $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\overset{\text{O}}{\parallel}\text{C}-\text{NH}-\text{(CH}_2\text{CH}_2\text{O})_4-\text{CH}_2\text{CH}_2\text{CH}_2\overset{\text{O}}{\parallel}\text{CH}$<br><p>mPEG Butyraldehyde Derivative</p>   | $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\overset{\text{O}}{\parallel}\text{C}-\text{NH}-\text{(CH}_2\text{CH}_2\text{O})_4-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}\text{CH}-\text{NH}-\text{F8}$<br><p>Secondary Amine Linkage</p>   |
| $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\overset{\text{O}}{\parallel}\text{C}-\text{NH}-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\overset{\text{O}}{\parallel}\text{CH}$<br><p>Branched mPEG2 Butyraldehyde</p>                            | $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\overset{\text{O}}{\parallel}\text{C}-\text{NH}-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}\text{CH}-\text{NH}-\text{(CH}_2\text{CH}_2\text{O})_4-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}\text{CH}-\text{NH}-\text{F8}$<br><p>Secondary Amine Linkage</p> |

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| Polymeric Reagent  | Corresponding Conjugate   |
|--|---|
| $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\overset{\text{OCH}_2\text{CH}_3}{\underset{ }{\text{O}}}-\text{CH}_2\text{CH}-\text{OCH}_2\text{CH}_3$<br>mPEG Acetal   | $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\text{NH}-\text{F8}$<br>Secondary Amine Linkage   |
| $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}\text{C}-\text{N}-\text{Cyclohexanone}-\text{O}$<br>mPEG Piperidone   | $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}\text{C}-\text{N}-\text{Cyclohexanone}-\text{NH}-\text{F8}$<br>Secondary Amine Linkage<br>(to a secondary carbon)  |
| $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-(\text{CH}_2)_{2-5}-\overset{\text{O}}{\parallel}\text{C}-\text{CH}_3$<br>mPEG Methylketone   | $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\overset{\text{NH}-\text{F8}}{\underset{ }{\text{O}}}-\text{O}-(\text{CH}_2)_{2-5}-\text{CH}-\text{CH}_3$<br>secondary amine linkage<br>(to a secondary carbon)   |
| $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}\text{C}-\text{N}-\text{Cyclohexanone}-\text{O}$<br>mPEG "Linkerless" Maleimide<br>(under certain reaction conditions such as pH > 8)   | $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}\text{C}-\text{N}-\text{Cyclohexanone}-\text{NH}-\text{F8}$<br>Secondary Amine Linkage   |
| $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\text{NH}-\overset{\text{O}}{\parallel}\text{C}-\text{CH}_2\text{CH}_2-\text{N}-\text{Cyclohexanone}-\text{O}$<br>mPEG Maleimide Derivative<br>(under certain reaction conditions such as pH > 8)  | $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\text{NH}-\overset{\text{O}}{\parallel}\text{C}-\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}\text{C}-\text{N}-\text{Cyclohexanone}-\text{NH}-\text{F8}$<br>Secondary Amine Linkage  |
| $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}\text{C}-\text{NH}-\text{CH}_2\text{CH}_2-\text{NH}-\overset{\text{O}}{\parallel}\text{C}-\text{CH}_2\text{CH}_2-\text{N}-\text{Cyclohexanone}-\text{O}$<br>mPEG Maleimide Derivative<br>(under certain reaction conditions such as pH > 8) | $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}\text{C}-\text{NH}-\text{CH}_2\text{CH}_2-\text{NH}-\overset{\text{O}}{\parallel}\text{C}-\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}\text{C}-\text{N}-\text{Cyclohexanone}-\text{NH}-\text{F8}$<br>Secondary Amine Linkage |

| Polymeric Reagent   | Corresponding Conjugate   |
|---|---|
|  <p>mPEG Forked Maleimide Derivative<br/>(under certain reaction conditions such as pH &gt; 8)</p>     |   |
|  <p>branched mPEG2 Maleimide Derivative<br/>(under certain reaction conditions such as pH &gt; 8)</p> |  <p>Secondary Amine Linkages<br/>Secondary Amine Linkage</p> |

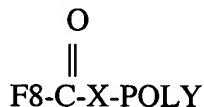
[0109] Conjugation of a polymeric reagent to an amino group of a Factor VIII moiety can be accomplished by one of ordinary skill in the art without undue experimentation. Typical of one approach is a reductive amination reaction used, for example, to conjugate a primary amine of a Factor VIII moiety with a polymer functionalized with a ketone, aldehyde or hydrated forms thereof (e.g., ketone hydrate, aldehyde hydrate). In this approach, the primary amine from the Factor VIII moiety reacts with the carbonyl group of the aldehyde or ketone (or the corresponding hydroxy-containing group of a hydrated aldehyde or ketone), thereby forming a Schiff base. The Schiff base, in turn, can then be reductively converted to a stable conjugate through use of a reducing agent such as sodium borohydride. Selective reactions (e.g., at the N-terminus are possible) are possible, particularly with a polymer functionalized with a ketone or an alpha-methyl branched aldehyde and/or under specific reaction conditions (e.g., reduced pH).

[0110] Preferred amine groups in Factor VIII that can serve as a site for attaching a polymer include those amine groups found within the following lysine residues: Lys<sup>493</sup>, Lys<sup>496</sup>,

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Lys<sup>499</sup>, Lys<sup>1804</sup>, Lys<sup>1808</sup>, Lys<sup>1813</sup>, Lys<sup>1818</sup>, Lys<sup>2183</sup>, Lys<sup>2207</sup>, Lys<sup>2227</sup>, Lys<sup>2236</sup>, with Lys<sup>496</sup>, Lys<sup>1804</sup>, and Lys<sup>1808</sup> being particularly preferred. Numbering corresponds to the sequence provided in SEQ. ID. NO. 2. As stated above, the amine group corresponding to each of these lysine residues in a protein other than human Factor VIII can serve as a useful site for conjugation. In addition, the N-terminus of any Factor VIII moiety that is a protein can serve as a polymeric attachment site.

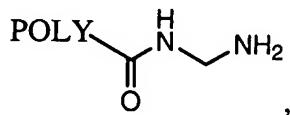
[0111] Carboxyl groups represent another functional group that can serve as a point of attachment on the Factor VIII moiety. Structurally, the conjugate will comprise the following:



where F8 and the adjacent carbonyl group corresponds to the carboxyl-containing Factor VIII moiety, X is a linkage, preferably a heteroatom selected from O, N(H), and S, and POLY is a water-soluble polymer such as PEG, optionally terminating in an end-capping moiety.

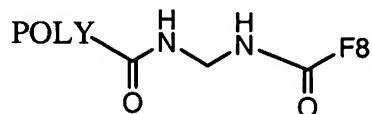
[0112] The C(O)-X linkage results from the reaction between a polymeric derivative bearing a terminal functional group and a carboxyl-containing Factor VIII moiety. As discussed above, the specific linkage will depend on the type of functional group utilized. If the polymer is end-functionalized or "activated" with a hydroxyl group, the resulting linkage will be a carboxylic acid ester and X will be O. If the polymer backbone is functionalized with a thiol group, the resulting linkage will be a thioester and X will be S. When certain multi-arm, branched or forked polymers are employed, the C(O)X moiety, and in particular the X moiety, may be relatively more complex and may include a longer linkage structure.

[0113] Water-soluble derivatives containing a hydrazide moiety are also useful for conjugation at carboxyl groups. An example of such a derivative includes a polymer having the following structure:



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which, upon conjugation with a Factor VIII moiety, has the following structure:



where F8 is the Factor VIII moiety following conjugation and POLY is a water-soluble polymer such as PEG, optionally terminating in an end-capping moiety.

**[0114]** Thiol groups contained within the Factor VIII moiety can serve as effective sites of attachment for the water-soluble polymer. In particular, cysteine residues provide thiol groups when the Factor VIII moiety is a protein. The thiol groups in such cysteine residues can then be reacted with an activated PEG that is specific for reaction with thiol groups, *e.g.*, an N-maleimidyl polymer or other derivative, as described in U.S. Patent No. 5,739,208 and in International Patent Publication No. WO 01/62827.

**[0115]** Specific examples, along with the corresponding conjugate, are provided in Table 2, below. In the table, the variable (n) represents the number of repeating monomeric units and "-S-F8" represents the Factor VIII moiety following conjugation to the water-soluble polymer. While each polymeric portion presented in Table 1 terminates in a "CH<sub>3</sub>" group, other groups (such as H and benzyl) can be substituted therefor.

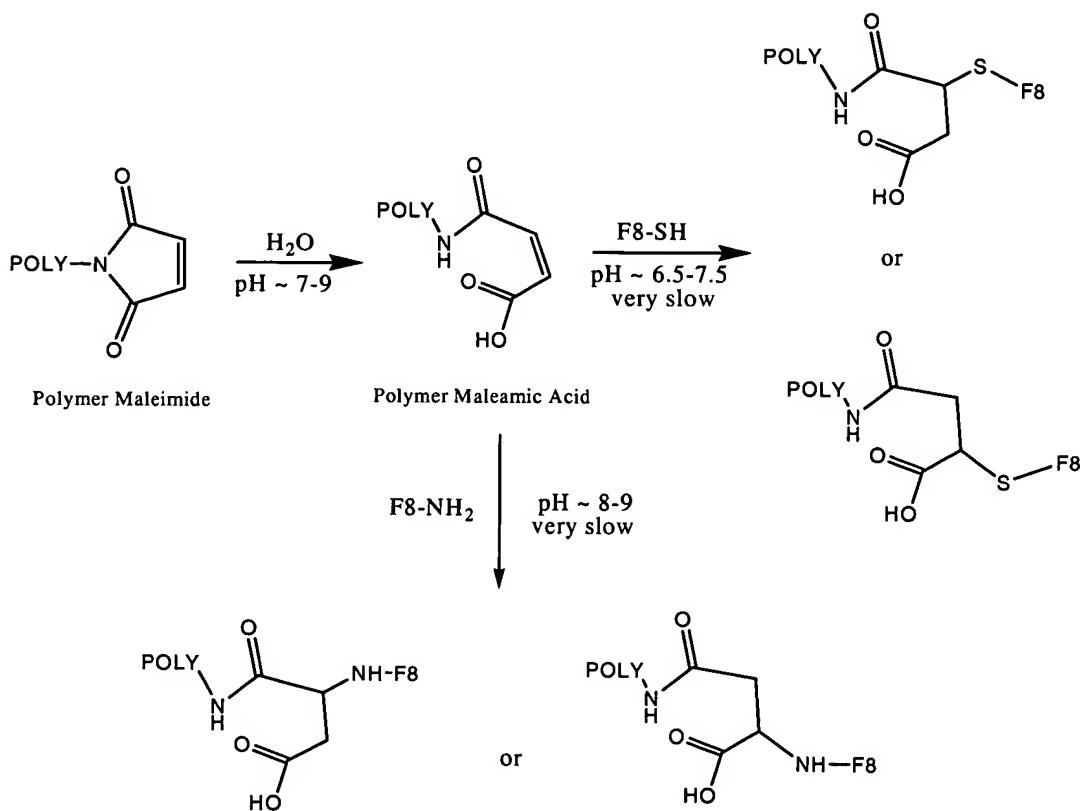
Table 2

Thiol-Specific Polymeric Reagents and the Factor VIII Moiety Conjugate Formed Therefrom

| Polymeric Reagent   | Corresponding Conjugate   |
|---|---|
| $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\text{N}$<br>mPEG "Linkerless" Maleimide  | $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\text{N}$<br>Thioether Linkage  |
| $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\text{NH}-\text{C}(=\text{O})-\text{CH}_2\text{CH}_2-\text{N}$<br>mPEG Maleimide Derivative | $\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\text{NH}-\text{C}(=\text{O})-\text{CH}_2\text{CH}_2-\text{N}$<br>Thioether Linkage |

| Polymeric Reagent  | Corresponding Conjugate   |
|--|---------------------------|
| <p><b>mPEG Maleimide Derivative</b></p>                  | <p>Thioether Linkage</p>  |
| <p><b>mPEG Forked Maleimide Derivative</b></p>           | <p>Thioether Linkage</p>  |
| <p><b>branched mPEG2 Maleimide Derivative</b></p>        | <p>Thioether Linkage</p>  |
| <p><b>Branched mPEG2 Forked Maleimide Derivative</b></p> | <p>Thioether Linkages</p> |
| <p><b>mPEG vinyl sulfone</b></p>                         | <p>Thioether Linkage</p>  |
| <p><b>mPEG thiol</b></p>                                 | <p>Disulfide Linkage</p>  |

[0116] With respect to conjugates formed from water-soluble polymers bearing one or more maleimide functional groups (regardless of whether the maleimide reacts with an amine or thiol group on the Factor VIII moiety), the corresponding maleamic acid form(s) of the water-soluble polymer can also react with the Factor VIII moiety. Under certain conditions (*e.g.*, a pH of about 7-9 and in the presence of water), the maleimide ring will "open" to form the corresponding maleamic acid. The maleamic acid, in turn, can react with an amine or thiol group of a Factor VIII moiety. Exemplary maleamic acid-based reactions are schematically shown below. POLY represents the water-soluble polymer, and F8 represents the Factor VIII moiety.



[0117] A representative conjugate in accordance with the invention can have the following structure:



wherein **POLY** is a water-soluble polymer, **L** is an optional linker, **Z** is a heteroatom selected from the group consisting of O, NH, and S, and **Y** is selected from the group consisting of C<sub>2-10</sub>

alkyl, C<sub>2</sub>-10 substituted alkyl, aryl, and substituted aryl. Polymeric reagents that can be reacted with a Factor VIII moiety and result in this type of conjugate are described in copending application filed on January 6, 2004, entitled "Thiol Selective Water Soluble Polymer Derivatives," and assigned U.S. Serial No. 10/753,047.

[0118] Preferred thiol groups in Factor VIII that can serve as a site for attaching a polymeric reagent include those thiol groups found within the following cysteine residues: Cys<sup>248</sup>, Cys<sup>310</sup>, Cys<sup>329</sup>, Cys<sup>630</sup>, Cys<sup>692</sup>, Cys<sup>711</sup>, Cys<sup>1899</sup>, Cys<sup>1903</sup>, and Cys<sup>2000</sup>, with Cys<sup>630</sup>, Cys<sup>711</sup>, and Cys<sup>1903</sup>, being particularly preferred. Numbering corresponds to the sequence provided in SEQ. ID. NO. 2.

[0119] With respect to polymeric reagents, those described here and elsewhere can be purchased from commercial sources (*e.g.*, Nektar Therapeutics, Huntsville AL). In addition, methods for preparing the polymeric reagents are described in the literature.

[0120] Typically, although not necessarily, the linkage between the Factor VIII moiety and the polymeric reagent includes one or more atoms such as one or more of carbon, nitrogen, sulfur, and combinations thereof. Preferably, the linkage comprises an amide, secondary amine, carbamate, thioether, or disulfide group. Optionally, additional atoms can connect the linkage to the chain of repeating monomers within the polymeric reagent. Nonlimiting examples of specific series of atoms connecting the Factor VIII moiety to the chain of repeating monomers include those selected from the group consisting of -O-, -S-, -S-S-, -C(O)-, -O-C(O)-, -C(O)-O-, -C(O)-NH-, -NH-C(O)-NH-, -O-C(O)-NH-, -C(S)-, -CH<sub>2</sub>-, -CH<sub>2</sub>-CH<sub>2</sub>-, -CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-, -O-CH<sub>2</sub>-, -CH<sub>2</sub>-O-, -O-CH<sub>2</sub>-CH<sub>2</sub>-, -CH<sub>2</sub>-O-CH<sub>2</sub>-, -CH<sub>2</sub>-CH<sub>2</sub>-O-, -O-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-, -CH<sub>2</sub>-O-CH<sub>2</sub>-CH<sub>2</sub>-, -CH<sub>2</sub>-CH<sub>2</sub>-O-CH<sub>2</sub>-, -O-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-, -CH<sub>2</sub>-O-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-, -CH<sub>2</sub>-CH<sub>2</sub>-O-CH<sub>2</sub>-CH<sub>2</sub>-, -CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-O-CH<sub>2</sub>-, -CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-O-, -C(O)-NH-CH<sub>2</sub>-, -C(O)-NH-CH<sub>2</sub>-CH<sub>2</sub>-, -CH<sub>2</sub>-C(O)-NH-CH<sub>2</sub>-CH<sub>2</sub>-, -CH<sub>2</sub>-CH<sub>2</sub>-C(O)-NH-, -C(O)-NH-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-, -CH<sub>2</sub>-C(O)-NH-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-, -CH<sub>2</sub>-CH<sub>2</sub>-C(O)-NH-CH<sub>2</sub>-, -CH<sub>2</sub>-CH<sub>2</sub>-C(O)-NH-CH<sub>2</sub>-, -C(O)-NH-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-, -CH<sub>2</sub>-C(O)-O-CH<sub>2</sub>-, -CH<sub>2</sub>-CH<sub>2</sub>-C(O)-O-CH<sub>2</sub>-, -C(O)-O-CH<sub>2</sub>-CH<sub>2</sub>-, -NH-C(O)-CH<sub>2</sub>-,

-CH<sub>2</sub>-NH-C(O)-CH<sub>2</sub>-, -CH<sub>2</sub>-CH<sub>2</sub>-NH-C(O)-CH<sub>2</sub>-, -NH-C(O)-CH<sub>2</sub>-CH<sub>2</sub>-,  
-CH<sub>2</sub>-NH-C(O)-CH<sub>2</sub>-CH<sub>2</sub>-, -CH<sub>2</sub>-CH<sub>2</sub>-NH-C(O)-CH<sub>2</sub>-CH<sub>2</sub>-, -C(O)-NH-CH<sub>2</sub>-,  
-C(O)-NH-CH<sub>2</sub>-CH<sub>2</sub>-, -O-C(O)-NH-CH<sub>2</sub>-, -O-C(O)-NH-CH<sub>2</sub>-CH<sub>2</sub>-,  
-O-C(O)-NH-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-, -NH-CH<sub>2</sub>-, -NH-CH<sub>2</sub>-CH<sub>2</sub>-, -CH<sub>2</sub>-NH-CH<sub>2</sub>-, -CH<sub>2</sub>-CH<sub>2</sub>-NH-CH<sub>2</sub>-,  
-C(O)-CH<sub>2</sub>-, -C(O)-CH<sub>2</sub>-CH<sub>2</sub>-, -CH<sub>2</sub>-C(O)-CH<sub>2</sub>-, -CH<sub>2</sub>-CH<sub>2</sub>-C(O)-CH<sub>2</sub>-,  
-CH<sub>2</sub>-CH<sub>2</sub>-C(O)-CH<sub>2</sub>-CH<sub>2</sub>-, -CH<sub>2</sub>-CH<sub>2</sub>-C(O)-, -CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-C(O)-NH-CH<sub>2</sub>-CH<sub>2</sub>-NH-,  
-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-C(O)-NH-CH<sub>2</sub>-CH<sub>2</sub>-NH-C(O)-,  
-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-C(O)-NH-CH<sub>2</sub>-CH<sub>2</sub>-NH-C(O)-CH<sub>2</sub>-,  
-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-C(O)-NH-CH<sub>2</sub>-CH<sub>2</sub>-NH-C(O)-CH<sub>2</sub>-CH<sub>2</sub>-,  
-O-C(O)-NH-[CH<sub>2</sub>]<sub>0-6</sub>-(OCH<sub>2</sub>CH<sub>2</sub>)<sub>0-2</sub>-, -C(O)-NH-(CH<sub>2</sub>)<sub>1-6</sub>-NH-C(O)-,  
-NH-C(O)-NH-(CH<sub>2</sub>)<sub>1-6</sub>-NH-C(O)-, -O-C(O)-CH<sub>2</sub>-, -O-C(O)-CH<sub>2</sub>-CH<sub>2</sub>-, and  
-O-C(O)-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-.

**[0121]** The conjugates are typically part of a composition. Generally, the composition comprises a plurality of conjugates, preferably although not necessarily, each having one to three water-soluble polymers covalently attached to one Factor VIII moiety. The compositions, however, can also comprise other conjugates having four, five, six, seven, eight or more polymers attached to any given moiety having Factor VIII activity. In addition, the invention includes instances wherein the composition comprises a plurality of conjugates, each conjugate comprising one water-soluble polymer covalently attached to one Factor VIII moiety, as well as compositions comprising two, three, four, five, six, seven, eight, or more water-soluble polymers covalently attached to one Factor VIII moiety.

**[0122]** Control of the desired number of polymers for any given moiety can be achieved by selecting the proper polymeric reagent, the ratio of polymeric reagent to the Factor VIII moiety, temperature, pH conditions, and other aspects of the conjugation reaction. In addition, reduction or elimination of the undesired conjugates (*e.g.*, those conjugates having four or more attached polymers) can be achieved through purification means.

**[0123]** For example, the polymer-Factor VIII moiety conjugates can be purified to obtain/isolate different conjugated species. Specifically, the product mixture can be purified to obtain an average of anywhere from one, two, three, four, five or more PEGs per Factor VIII moiety, typically one, two or three PEGs per Factor VIII moiety. The strategy for purification of

the final conjugate reaction mixture will depend upon a number of factors, including, for example, the molecular weight of the polymeric reagent employed, the particular Factor VIII moiety, the desired dosing regimen, and the residual activity and *in vivo* properties of the individual conjugate(s).

[0124] If desired, conjugates having different molecular weights can be isolated using gel filtration chromatography and/or ion exchange chromatography. That is to say, gel filtration chromatography is used to fractionate differently numbered polymer-to-Factor VIII moiety ratios (*e.g.*, 1-mer, 2-mer, 3-mer, and so forth, wherein "1-mer" indicates 1 polymer to Factor VIII moiety, "2-mer" indicates two polymers to Factor VIII moiety, and so on) on the basis of their differing molecular weights (where the difference corresponds essentially to the average molecular weight of the water-soluble polymer portion). For example, in an exemplary reaction where a 100,000 Dalton protein is randomly conjugated to a polymeric reagent having a molecular weight of about 20,000 Daltons, the resulting reaction mixture may contain unmodified protein (having a molecular weight of about 100,000 Daltons), monoPEGylated protein (having a molecular weight of about 120,000 Daltons), diPEGylated protein (having a molecular weight of about 140,000 Daltons), and so forth.

[0125] While this approach can be used to separate PEG and other polymer-Factor VIII moiety conjugates having different molecular weights, this approach is generally ineffective for separating positional isomers having different polymer attachment sites within the Factor VIII moiety. For example, gel filtration chromatography can be used to separate from each other mixtures of PEG 1-mers, 2-mers, 3-mers, and so forth, although each of the recovered PEG-mer compositions may contain PEGs attached to different reactive amino groups (*e.g.*, lysine residues) within Factor VIII moiety.

[0126] Gel filtration columns suitable for carrying out this type of separation include Superdex™ and Sephadex™ columns available from Amersham Biosciences (Piscataway, NJ). Selection of a particular column will depend upon the desired fractionation range desired. Elution is generally carried out using a suitable buffer, such as phosphate, acetate, or the like. The collected fractions may be analyzed by a number of different methods, for example, (i) optical density (OD) at 280 nm for protein content, (ii) bovine serum albumin (BSA) protein analysis, (iii) iodine testing for PEG content (Sims *et al.* (1980) *Anal. Biochem.*, 107:60-63), and

(iv) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE), followed by staining with barium iodide.

[0127] Separation of positional isomers is carried out by reverse phase chromatography using a reverse phase-high performance liquid chromatography (RP-HPLC) C18 column (Amersham Biosciences or Vydac) or by ion exchange chromatography using an ion exchange column, *e.g.*, a Sepharose™ ion exchange column available from Amersham Biosciences. Either approach can be used to separate polymer-active agent isomers having the same molecular weight (positional isomers).

[0128] The compositions are preferably substantially free of proteins that do not have Factor VIII activity. In addition, the compositions preferably are substantially free of all other noncovalently attached water-soluble polymers. Moreover, at least one species of conjugate in the composition has at least one water-soluble water polymer attached to a moiety that transforms Factor X to Factor Xa. In some circumstances, however, the composition can contain a mixture of polymer-Factor VIII moiety conjugates and unconjugated Factor VIII.

[0129] Optionally, the composition of the invention further comprises a pharmaceutically acceptable excipient. If desired, the pharmaceutically acceptable excipient can be added to a conjugate to form a composition.

[0130] Exemplary excipients include, without limitation, those selected from the group consisting of carbohydrates, inorganic salts, antimicrobial agents, antioxidants, surfactants, buffers, acids, bases, and combinations thereof.

[0131] A carbohydrate such as a sugar, a derivatized sugar such as an alditol, aldonic acid, an esterified sugar, and/or a sugar polymer may be present as an excipient. Specific carbohydrate excipients include, for example: monosaccharides, such as fructose, maltose, galactose, glucose, D-mannose, sorbose, and the like; disaccharides, such as lactose, sucrose, trehalose, cellobiose, and the like; polysaccharides, such as raffinose, melezitose, maltodextrins, dextrans, starches, and the like; and alditols, such as mannitol, xylitol, maltitol, lactitol, xylitol, sorbitol (glucitol), pyranosyl sorbitol, myoinositol, and the like.

[0132] The excipient can also include an inorganic salt or buffer such as citric acid, sodium chloride, potassium chloride, sodium sulfate, potassium nitrate, sodium phosphate monobasic, sodium phosphate dibasic, and combinations thereof.

[0133] The composition can also include an antimicrobial agent for preventing or deterring microbial growth. Nonlimiting examples of antimicrobial agents suitable for the present invention include benzalkonium chloride, benzethonium chloride, benzyl alcohol, cetylpyridinium chloride, chlorobutanol, phenol, phenylethyl alcohol, phenylmercuric nitrate, thimersol, and combinations thereof.

[0134] An antioxidant can be present in the composition as well. Antioxidants are used to prevent oxidation, thereby preventing the deterioration of the conjugate or other components of the preparation. Suitable antioxidants for use in the present invention include, for example, ascorbyl palmitate, butylated hydroxyanisole, butylated hydroxytoluene, hypophosphorous acid, monothioglycerol, propyl gallate, sodium bisulfite, sodium formaldehyde sulfoxylate, sodium metabisulfite, and combinations thereof.

[0135] A surfactant can be present as an excipient. Exemplary surfactants include: polysorbates, such as "Tween 20" and "Tween 80," and pluronics such as F68 and F88 (both of which are available from BASF, Mount Olive, New Jersey); sorbitan esters; lipids, such as phospholipids such as lecithin and other phosphatidylcholines, phosphatidylethanolamines (although preferably not in liposomal form), fatty acids and fatty esters; steroids, such as cholesterol; and chelating agents, such as EDTA, zinc and other such suitable cations.

[0136] Acids or bases can be present as an excipient in the composition. Nonlimiting examples of acids that can be used include those acids selected from the group consisting of hydrochloric acid, acetic acid, phosphoric acid, citric acid, malic acid, lactic acid, formic acid, trichloroacetic acid, nitric acid, perchloric acid, phosphoric acid, sulfuric acid, fumaric acid, and combinations thereof. Examples of suitable bases include, without limitation, bases selected from the group consisting of sodium hydroxide, sodium acetate, ammonium hydroxide, potassium hydroxide, ammonium acetate, potassium acetate, sodium phosphate, potassium phosphate, sodium citrate, sodium formate, sodium sulfate, potassium sulfate, potassium fumerate, and combinations thereof.

[0137] The amount of the conjugate (*i.e.*, the conjugate formed between the active agent and the polymeric reagent) in the composition will vary depending on a number of actors, but will optimally be a therapeutically effective dose when the composition is stored in a unit dose container (*e.g.*, a vial). In addition, the pharmaceutical preparation can be housed in a syringe. A therapeutically effective dose can be determined experimentally by repeated administration of increasing amounts of the conjugate in order to determine which amount produces a clinically desired endpoint.

[0138] The amount of any individual excipient in the composition will vary depending on the activity of the excipient and particular needs of the composition. Typically, the optimal amount of any individual excipient is determined through routine experimentation, *i.e.*, by preparing compositions containing varying amounts of the excipient (ranging from low to high), examining the stability and other parameters, and then determining the range at which optimal performance is attained with no significant adverse effects.

[0139] Generally, however, the excipient will be present in the composition in an amount of about 1% to about 99% by weight, preferably from about 5% to about 98% by weight, more preferably from about 15 to about 95% by weight of the excipient, with concentrations less than 30% by weight most preferred.

[0140] These foregoing pharmaceutical excipients along with other excipients are described in "Remington: The Science & Practice of Pharmacy", 19<sup>th</sup> ed., Williams & Williams, (1995), the "Physician's Desk Reference", 52<sup>nd</sup> ed., Medical Economics, Montvale, NJ (1998), and Kibbe, A.H., Handbook of Pharmaceutical Excipients, 3<sup>rd</sup> Edition, American Pharmaceutical Association, Washington, D.C., 2000.

[0141] The compositions encompass all types of formulations and in particular those that are suited for injection, *e.g.*, powders or lyophilates that can be reconstituted as well as liquids. Examples of suitable diluents for reconstituting solid compositions prior to injection include bacteriostatic water for injection, dextrose 5% in water, phosphate-buffered saline, Ringer's solution, saline, sterile water, deionized water, and combinations thereof. With respect to liquid pharmaceutical compositions, solutions and suspensions are envisioned.

[0142] The compositions of the present invention are typically, although not necessarily, administered via injection and are therefore generally liquid solutions or suspensions immediately prior to administration. The pharmaceutical preparation can also take other forms such as syrups, creams, ointments, tablets, powders, and the like. Other modes of administration are also included, such as pulmonary, rectal, transdermal, transmucosal, oral, intrathecal, subcutaneous, intra-arterial, and so forth.

[0143] The invention also provides a method for administering a conjugate as provided herein to a patient suffering from a condition that is responsive to treatment with conjugate. The method comprises administering, generally via injection, a therapeutically effective amount of the conjugate (preferably provided as part of a pharmaceutical composition). As previously described, the conjugates can be administered injected parenterally by intravenous injection, or less preferably by intramuscular or by subcutaneous injection. Suitable formulation types for parenteral administration include ready-for-injection solutions, dry powders for combination with a solvent prior to use, suspensions ready for injection, dry insoluble compositions for combination with a vehicle prior to use, and emulsions and liquid concentrates for dilution prior to administration, among others.

[0144] The method of administering may be used to treat any condition that can be remedied or prevented by administration of the conjugate. Those of ordinary skill in the art appreciate which conditions a specific conjugate can effectively treat. For example, the conjugates can be used to treat individuals suffering from hemophilia A. In addition, the conjugates are suited for use as a prophylactic against uncontrolled bleeding, optionally in patients not suffering from hemophilia. Thus, for example, the conjugate can be administered to a patient at risk for uncontrolled bleeding prior to surgery.

[0145] The actual dose to be administered will vary depend upon the age, weight, and general condition of the subject as well as the severity of the condition being treated, the judgment of the health care professional, and conjugate being administered. Therapeutically effective amounts are known to those skilled in the art and/or are described in the pertinent reference texts and literature. Generally, a therapeutically effective amount will range from about 0.001 mg to 100 mg, preferably in doses from 0.01 mg/day to 75 mg/day, and more preferably in doses from 0.10 mg/day to 50 mg/day.

[0146] The unit dosage of any given conjugate (again, preferably provided as part of a pharmaceutical preparation) can be administered in a variety of dosing schedules depending on the judgment of the clinician, needs of the patient, and so forth. The specific dosing schedule will be known by those of ordinary skill in the art or can be determined experimentally using routine methods. Exemplary dosing schedules include, without limitation, administration five times a day, four times a day, three times a day, twice daily, once daily, three times weekly, twice weekly, once weekly, twice monthly, once monthly, and any combination thereof. Once the clinical endpoint has been achieved, dosing of the composition is halted.

[0147] One advantage of administering certain conjugates of the present invention is that individual water-soluble polymer portions can be cleaved off. Such a result is advantageous when clearance from the body is potentially a problem because of the polymer size. Optimally, cleavage of each water-soluble polymer portion is facilitated through the use of physiologically cleavable and/or enzymatically degradable linkages such as urethane, amide, carbonate or ester-containing linkages. In this way, clearance of the conjugate (via cleavage of individual water-soluble polymer portions) can be modulated by selecting the polymer molecular size and the type functional group that would provide the desired clearance properties. One of ordinary skill in the art can determine the proper molecular size of the polymer as well as the cleavable functional group. For example, one of ordinary skill in the art, using routine experimentation, can determine a proper molecular size and cleavable functional group by first preparing a variety of polymer derivatives with different polymer weights and cleavable functional groups, and then obtaining the clearance profile (e.g., through periodic blood or urine sampling) by administering the polymer derivative to a patient and taking periodic blood and/or urine sampling. Once a series of clearance profiles have been obtained for each tested conjugate, a suitable conjugate can be identified.

[0148] It is to be understood that while the invention has been described in conjunction with the preferred specific embodiments thereof, that the foregoing description as well as the examples that follow are intended to illustrate and not limit the scope of the invention. Other aspects, advantages and modifications within the scope of the invention will be apparent to those skilled in the art to which the invention pertains.

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[0149] All articles, books, patents and other publications referenced herein are hereby incorporated by reference in their entireties.

## EXPERIMENTAL

[0150] The practice of the invention will employ, unless otherwise indicated, conventional techniques of organic synthesis and the like, which are within the skill of the art. Such techniques are fully explained in the literature. See, for example, J. March, Advanced Organic Chemistry: Reactions Mechanisms and Structure, 4th Ed. (New York: Wiley-Interscience, 1992), *supra*.

[0151] In the following examples, efforts have been made to ensure accuracy with respect to numbers used (*e.g.*, amounts, temperatures, etc.) but some experimental error and deviation should be accounted for. Unless indicated otherwise, temperature is in degrees C and pressure is at or near atmospheric pressure at sea level.

[0152] ABBREVIATIONS:

[0153] DCM dichloromethane

[0154] mPEG-SPA mPEG-succinimidyl propionate

[0155] mPEG-SBA mPEG-succinimidyl butanoate

[0156] mPEG-OPSS mPEG-orthopyridyl-disulfide

[0157] mPEG-MAL mPEG-maleimide,  $\text{CH}_3\text{O}-(\text{CH}_2\text{CH}_2\text{O})_n-\text{CH}_2\text{CH}_2\text{-MAL}$

[0158] mPEG-SMB mPEG-succinimidyl  $\alpha$ -methylbutanoate,  
 $\text{CH}_3\text{O}-(\text{CH}_2\text{CH}_2\text{O})_n-\text{CH}_2\text{CH}_2\text{-CH}(\text{CH}_3)\text{-C(O)-O-succinimide}$

[0159] mPEG-ButyrALD

$\text{CH}_3\text{O}-(\text{CH}_2\text{CH}_2\text{O})_n-\text{CH}_2\text{CH}_2\text{-O-C(O)-NH-(CH}_2\text{CH}_2\text{O)}_4\text{CH}_2\text{CH}_2\text{CH}_2\text{C(O)H}$

[0160] mPEG-PIP  $\text{CH}_3\text{O}-(\text{CH}_2\text{CH}_2\text{O})_n-\text{CH}_2\text{CH}_2\text{-C(O)-piperidin-4-one}$

[0161] SUC succinimide or succinimidyl

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|        |                             |   |
|--------|-----------------------------|---|
| [0162] | NaCNBH <sub>3</sub>         | sodium cyanoborohydride                                   |
| [0163] | HCl                         | hydrochloric acid   |
| [0164] | HEPES                       | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid        |
| [0165] | NMR                         | nuclear magnetic resonance                                |
| [0166] | DCC                         | 1,3-dicyclohexylcarbodiimide                              |
| [0167] | DI                          | deionized   |
| [0168] | MW                          | molecular weight  |
| [0169] | r.t.                        | room temperature  |
| [0170] | K or kDa                    | kilodaltons   |
| [0171] | SEC                         | Size exclusion chromatography                             |
| [0172] | HPLC                        | high performance liquid chromatography                    |
| [0173] | FPLC                        | fast protein liquid chromatography                        |
| [0174] | SDS-PAGE                    | sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| [0175] | MALDI-TOF<br>Time-of-Flight | Matrix Assisted Laser Desorption Ionization               |

[0176] MATERIALS:

[0177] All PEG reagents referred to in the appended examples are commercially available unless otherwise indicated.

[0178] mPEG-succinimidyl propionate, mPEG-SPA, molecular weight, 30K ( $M_n=31.3$  kDa, Nektar Therapeutics)

[0179] mPEG-orthopyridyl-disulfide, mPEG-OPSS, molecular weight, 10K ( $M_n=10.3$  kDa, Nektar Therapeutics)

[0180] mPEG-maleimide, mPEG-MAL, molecular weight, 20K ( $M_n=21.8$  kDa, Nektar Therapeutics)

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- [0181] mPEG-maleimide, mPEG-MAL, molecular weight, 30K ( $M_n=31.4$  kDa, Nektar Therapeutics)
- [0182] mPEG-succinimidyl  $\alpha$ -methylbutanoate, mPEG-SMB, molecular weight, 30K ( $M_n=30.5$  kDa, Nektar Therapeutics)
- [0183] mPEG-butyraldehyde, mPEG-ButyrALD, molecular weight, 30K ( $M_n=31.5$  kDa, Nektar Therapeutics)
- [0184] L-Histidine, biotechnology performance certified (Sigma)
- [0185] HEPES, biotechnology performance certified, 99.5+% (Sigma)
- [0186] Calcium chloride, dihydrate, for molecular biology, 99% (Sigma)
- [0187] Sodium chloride, for molecular biology (Sigma)
- [0188] Tween 80, Sigma Ultra, (Sigma)
- [0189] Ethyl alcohol, USP, Absolute-200 Proof (AAPER)
- [0190] Polyethylene glycol, MW 3,350, SigmaUltra (Sigma)
- [0191] Slide-A-Lyzer Dialysis Cassette, 0.5-3ml, or 3-12ml capacity (Pierce)
- [0192] Acetic acid, A.C.S. reagent, 99.7+% (Aldrich)
- [0193] 1N Acetic acid, volumetric standard (VWR)
- [0194] 1N Sodium hydroxide, volumetric standard (J.T.Baker)
- [0195] Sodium cyanoborohydride, 95% (Aldrich)
- [0196] Tris/glycine/SDS, 10x, protein electrophoresis buffer (Bio-Rad)
- [0197] Laemmli sample buffer (Bio-Rad)
- [0198] SigmaMarker, low range (M.W. 6,500-66,000) (Sigma)
- [0199] SigmaMarker, high range (M.W. 36,000-205,000) (Sigma)
- [0200] 7.5% Tris-HCl ready gel (10-well, 30ul, Bio-Rad)
- [0201] GelCode blue stain reagent (Pierce)

[0202] METHODS (ANALYTICAL)

[0203] SEC-HPLC Analysis

[0204] Size exclusion chromatography (SEC) was performed on an Agilent 1100 HPLC system (Agilent). Samples were analyzed using a BIOSEP-SEC-S 4000 column (Phenomenex) and a mobile phase of 45 mM histidine, 4.5 mM calcium chloride, 0.36 M sodium chloride, 0.009%(v/v) Tween 80 and 10% ethyl alcohol, pH 6.7. The flow rate for the column was 0.3 ml/min. Eluted protein and PEG-protein conjugates were detected by UV at a wavelength of 280nm.

[0205] SDS-PAGE Analysis

[0206] Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using Mini-PROTEAN 3 Precast Gel Electrophoresis System (Bio-Rad). Samples were mixed with 2x Laemmli sample buffer, and were placed in a 95 °C water bath for ~ 5 minutes. Then, the prepared samples were loaded onto a 7.5% Tris-HCl ready gel and run for approximately 30 minutes at 200 V using Tris/glycine/SDS electrophoresis buffer.

[0207] OTHER METHODS

[0208] Purification of PEG-Factor VIII Conjugates

[0209] A Superose 6 HR 10/30, 24 ml gel filtration column (Amersham) was used with an FPLC system and AKTA prime system (Amersham) to purify the PEG-Factor VIII conjugates in Examples 6-11. The flow rate was 0.3 ml/min and the elution buffer was 50 mM Histidine, 0.5 M NaCl, 4.0 mM CaCl<sub>2</sub>, and 0.01% (w/v) Tween 80, pH 6.7.

[0210] Buffer exchange of Factor VIII Stock Solution

[0211] A Slide-A-Lyzer Dialysis Cassette (3-12 ml, 10,000 MWCO, Pierce) was removed from the protective pouch, and was soaked in MiliQ H<sub>2</sub>O for 15 minutes (water was changed every 5 minutes). The Factor VIII stock solution [0.398 mg/ml in 50 mM Histidine, 0.5

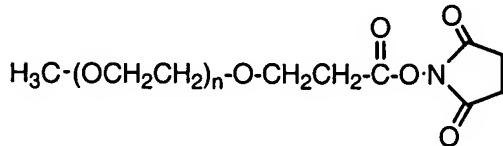
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M NaCl, 4.0 mM CaCl<sub>2</sub>, 0.1% (w/v) PEG 3,350, 0.01% (w/v) Tween 80, pH 6.7] was then transferred into the cassette cavity through one of the guide ports on the top of the gasket. The cassette was placed in a 1 L beaker of HEPES buffer [50 mM HEPES, 0.5 M NaCl, 5 mM CaCl<sub>2</sub>, 0.1% (w/v) PEG 3,350, 0.01% (v/v) Tween 80, pH 7.0] with a flotation buoy attached to the top of the cassette. The beaker was then placed on the stir plate to start the dialysis at 4 °C. The HEPES buffer was changed for four times at intervals of 2~3 hrs, and was then left in a cold room (4 °C) for overnight dialysis. After dialysis, the cassette chamber was injected with air and the dialyzed sample was withdrawn from the cassette. The concentration of Factor VIII in HEPES buffer was measured at UV 280nm from a SPECTRA max PLUS Spectrophotometer (Molecular Devices).

### Example 1

#### **PEGylation of B-domain Deleted Factor VIII with mPEG-SPA, 20K**

[0212] mPEG-Succinimidyl propionate having a molecular weight of 20,000 Daltons is obtained from Nektar Therapeutics, (Huntsville, AL). The basic structure of the polymer reagent is provided below:



[0213] B-domain deleted Factor VIII is dissolved in deionized water, to which is added triethylamine to raise the pH to 7.2-9. To the above solution is added a 1.5 to 10-fold molar excess of the PEG reagent, mPEG-SPA. The resulting mixture is stirred at room temperature for several hours.

[0214] The reaction mixture is analyzed by SDS-PAGE to determine the degree of PEGylation of the protein. The degree of PEGylation, 1-mer, 2 mers, etc., can also be determined by any of a number of analytical techniques appropriate for proteins of this size, such as light angle scattering. The displayed peaks for native and mono-PEGylated species differ by

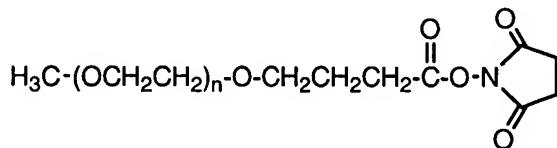
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approximately 20,000 Da. Increasing the ratio of PEG reagent to protein increases the degree of polyPEGylation, that is to say, the formation of 2-mers, 3-mers, etc.

**Example 2**

**PEGylation of B-domain Deleted Factor VIII with mPEG-SBA**

[0215] mPEG-Succinimidyl butanoate having a molecular weight of 10,000 daltons is obtained from Nektar Therapeutics, (Huntsville, AL). The basic structure of the polymer reagent is provided below:



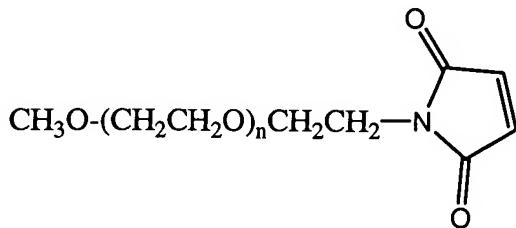
[0216] B-domain deleted Factor VIII is dissolved in deionized water, to which is added triethylamine to raise the pH to 7.2-9. To this solution is then added a 1.5 to 10-fold molar excess of mPEG-SBA. The resulting mixture is stirred at room temperature for several hours.

[0217] The reaction mixture is analyzed by SDS-PAGE to determine the degree of PEGylation of the protein.

**Example 3**

**PEGylation of B-domain Deleted Factor VIII with mPEG-MAL, 20K**

[0218] mPEG-Maleimide having a molecular weight of 20,000 daltons is obtained from Nektar Therapeutics, (Huntsville, AL). The basic structure of the polymer reagent is provided below:

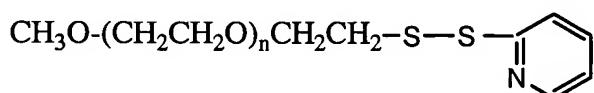


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[0219] B-domain deleted Factor VIII is dissolved in buffer. To this protein solution is added a 3-5 fold molar excess of mPEG-MAL. The mixture is stirred at room temperature under an inert atmosphere for several hours. The reaction mixture is analyzed and purified by HPLC to provide a mixture of conjugated species.

**Example 4**

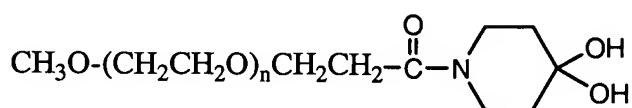
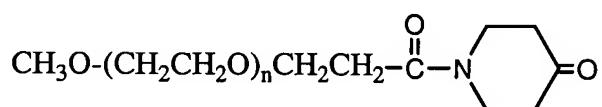
**PEGylation of B-domain Deleted Factor VIII with mPEG-OPSS, 20K**



[0220] The sulfhydryl-selective polymer reagent, mPEG-orthopyridyl-disulfide (structure shown above), having a molecular weight of 20,000, is obtained from Nektar Therapeutics (Huntsville, AL). A five-fold molar excess of mPEG-OPSS is added to B-domain deleted Factor VIII in a buffered solution. The reaction mixture is stirred for several hours at room temperature under an inert atmosphere to form the desired conjugate having a disulfide linkage connecting the polymer to the protein.

**Example 5**

**PEGylation of B-domain Deleted Factor VIII with mPEG-PIP, 5K**



[0221] The above polymeric reagent, shown as both the ketone and corresponding ketal, is prepared as described in Nektar Therapeutics' Provisional Patent Application No. 60/437,325, entitled "Polymer Derivatives and Conjugates Thereof."

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[0222] To prepare the above polymeric reagent, to a solution of methoxy-polyethylene glycol-succinimidyl propionate having a weight average molecular weight of 5,000 Daltons (1.0 g, 0.002 moles) in methylene chloride (20 ml), triethyl amine (0.084 ml, 0.006 moles) and 4-piperidone monohydrate hydrochloride (0.077g, 0.005 moles) are added. The reaction mixture is stirred at room temperature under a nitrogen atmosphere overnight and then purified prior to conjugation. Alternatively, the polymer reagent may be purchased from Nektar Therapeutics.

[0223] To effect conjugation, to a solution of B-domain deleted Factor VIII in aqueous buffer is added a 20-fold molar excess of mPEG-PIP, 5K. The resulting solution is placed on a Roto Mix™ orbital shaker (Thermolyne Corp., Dubuque, IA) set at slow speed to facilitate reaction at room temperature. After 15 minutes, aqueous NaCNBH<sub>3</sub> is added in an amount equal to a 50 fold-molar excess relative to the B-domain deleted Factor VIII. Aliquots are withdrawn at timed intervals from the reaction mixture and are analyzed by SDS-PAGE (using gels available from Bio-Rad Laboratories, Hercules, CA).

[0224] SDS-PAGE analysis indicates the presence of PEG derivatives of B-domain deleted Factor VIII having 1, 2, and 3 PEG moieties attached.

### Example 6

#### Conjugation of B-Domain Deleted Factor VIII with mPEG-SPA, 30K

[0225] Prior to conjugation, a buffer exchange for B-domain deleted Factor VIII (Factor VIII) was performed to replace histidine with HEPES.

[0226] mPEG-SPA, 30K, stored at -20 °C under argon, was warmed to ambient temperature. The warmed mPEG-SPA (2.2 mg) was dissolved in 0.022 ml of 2 mM HCl to provide a 10% solution of polymer reagent. The mPEG-SPA solution was quickly added to 3 ml of Factor VIII solution [0.412mg/ml in 50 mM HEPES, 0.5 M NaCl, 4.0 mM CaCl<sub>2</sub>, 0.1% (w/v) PEG 3,350, 0.01% (w/v) Tween 80, pH 7.0] and mixed well. After 30 minutes of reaction at room temperature, the reaction vial was transferred to a cold room (4 °C), and another 0.022 ml of mPEG-SPA solution was added to the reaction mixture, and mixed well. The pH was determined (pH 7.0±0.2). The molar ratio of mPEG-SPA to protein was 20:1. The final

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mPEG-SPA concentration was 1.445 mg/ml, and the final Factor VIII concentration was 0.406 mg/ml. The reaction was allowed to proceed overnight at 4 °C on Rotomix (slow speed, Thermolyne). The resulting conjugate was assigned identifier "pz041701."

[0227] The conjugate mixture was purified using gel filtration chromatography. A size exclusion chromatography method was developed for analyzing the reaction mixtures, and the final products. SDS-PAGE analysis was also used for the characterization of the samples.

[0228] CONJUGATE CHARACTERIZATION. The resulting conjugate mixture, prior to purification, was a mixture of Factor VII PEG-monomer (or 1-mer), dimer (or 2-mer) and trimer (or 3-mer), corresponding to the identifiers "pz041701 (low)," "pz041701 (medium)," and "pz041701 (high)" respectively, as determined by SEC. That is to say: "pz041701 (low)" corresponds to mostly Factor VIII mono-PEGylated species or Factor VIII having one PEG moiety attached thereto; "pz041701 (medium)" corresponds to primarily Factor VIII di-PEGylated species, that is to say, Factor VIII having two PEG moieties attached thereto; and "pz041701 (high)" corresponds mostly to Factor VIII having three PEG moieties attached thereto. The corresponding SEC plots are shown in FIGS. 1 and 2. FIG. 1 shows the SEC plot corresponding to fractions collected upon SEC of the Factor VIII reaction mixture. According to the size exclusion chromatography (SEC) results, the PEGylation yield of mPEG-SPA-30K-FVIII (pz041701 low) was ~ 39%. The PEGylation yield of mPEG30-SPA-30K-FVIII (pz041701 medium) was ~ 32%, and the PEGylation yield of mPEG-SPA-30K-FVIII (pz041701 high) was ~ 11%, with percentages based upon relative amounts compared to all species present in the resulting reaction mixture. The conjugate mixture was further purified by FPLC and analyzed by SDS-PAGE.

### Example 7

#### Conjugation of B-Domain Deleted Factor VIII with mPEG-MAL, 20K

[0229] Prior to the conjugation, a buffer exchange for B-domain deleted Factor VIII (Factor VIII) was performed to replace histidine with HEPES.

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[0230] mPEG-MAL, 20K, stored at -20 °C under argon, was warmed to ambient temperature. The warmed mPEG-MAL reagent (4.4 mg) was dissolved in 0.044 ml of HEPES buffer [50 mM HEPES, 0.15 M NaCl, 4.0 mM CaCl<sub>2</sub>, 0.01% (w/v) Tween 80, pH 7.0] to make a 10% mPEG-MAL solution. The mPEG-MAL solution was quickly added to 4 ml of Factor VIII solution [0.4324 mg/ml in 50 mM HEPES, 0.5 M NaCl, 4 mM CaCl<sub>2</sub>, 0.1% (w/v) PEG 3,350, 0.01% (w/v) Tween 80, pH 7.0] and mixed well. After 30 minutes of reaction at room temperature, the reaction vial was transferred to the cold room (4 °C), and another 0.044 ml of mPEG-MAL solution was added to the reaction mixture, followed by the addition of three more aliquots of 0.044 ml of mPEG-MAL solution over the course of two hours. The pH was determined (pH 7.0±0.2). The molar ratio of mPEG-MAL to protein was 100:1. The final mPEG-MAL concentration was 5.213 mg/ml, and the final Factor VIII concentration was 0.410 mg/ml. The reaction was allowed to proceed overnight at 4 °C on Rotomix (slow speed, Thermolyne). The conjugate was assigned identifier "pz061201."

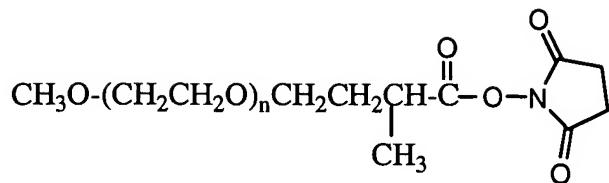
[0231] The conjugate mixture was purified using gel filtration chromatography. A size exclusion chromatography method was developed for analyzing the reaction mixtures, and the final products. SDS-PAGE analysis was also used for the characterization of the samples.

[0232] CONJUGATE CHARACTERIZATION (Mono-PEGylated product). According to the size exclusion chromatography (SEC) results, the PEGylation yield of monoPEGylated conjugate (1-mer) was ~ 33% (FIG. 3). The Factor VIII conjugate mixture fractions were combined and purified by FPLC, and then further purified by gel filtration chromatography. The pz061201 final product was analyzed by both SDS-PAGE and SEC, and the purity of the "pz061201" product was determined to be approximately 94% Factor VIII PEG monomer (*i.e.*, monopegylated Factor VIII), with ~ 6% Factor VIII PEG high-mers (FIG 4).

### Example 8

#### Conjugation of B-Domain Deleted Factor VIII with mPEG-SMB, 30K

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[0233] Prior to conjugation, a buffer exchange for B-domain deleted Factor VIII (Factor VIII) was performed to replace histidine with HEPES.

[0234] mPEG-SMB, 30K, stored at -20 °C under argon, was warmed to ambient temperature. The warmed mPEG-SMB (6.5 mg) was dissolved in 0.065 ml of 2mM HCl to form a 10% mPEG-SMB solution. The mPEG-SMB solution was quickly added to 4 ml of Factor VIII solution [0.435 mg/ml in 50 mM HEPES, 0.5 M NaCl, 5.0 mM CaCl<sub>2</sub>, 0.1% (w/v) PEG 3,350, 0.01% (v/v) Tween 80, pH 7.0] and mixed well. After 30 minutes of reaction at room temperature, the reaction vial was transferred to a cold room (4 °C). The pH was determined (pH 7.0±0.2). The molar ratio of mPEG-SMB to protein was 20:1. The final mPEG-SMB concentration was 1.599 mg/ml, and the final Factor VIII concentration was 0.428 mg/ml. The reaction was allowed to proceed for approximately 48 hrs at 4 °C on Rotomix (slow speed, Thermolyne), and was then quenched by the addition of acetic acid (99.7+%) to lower the pH to 6.0±0.3. The conjugate was assigned identifier "pz082501."

[0235] The conjugate mixture was purified using gel filtration chromatography. A size exclusion chromatography method was developed for analyzing the reaction mixtures, and the final products. SDS-PAGE analysis was also used for the characterization of the samples.

[0236] CONJUGATE CHARACTERIZATION: The mixture designated "pz082501" resulted from the PEGylation of Factor VIII with mPEG-SMB30K at pH 7.0±0.2. The conjugate mixture was purified and analyzed by SEC. Based upon the size exclusion chromatography (SEC) results, the PEGylation yield of pz082501, monoPEGylated conjugate (Factor VIII 1-mer) was ~41% (FIG. 5). The product mixture was further purified by FPLC and analyzed by SDS-PAGE and SEC. Characterization of the purified Factor VIII PEG conjugate product, pz082501, was ~95% mono-conjugated PEG Factor VIII with ~ 5% higher-mers.

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### Example 9

#### Conjugation of B-Domain Deleted Factor VIII with mPEG-OPSS, 10K

[0237] Prior to conjugation, a buffer exchange for B-domain deleted Factor VIII (Factor VIII) was performed to replace histidine with HEPES.

[0238] mPEG-OPSS, 10K, stored at -20 °C under argon, was warmed to ambient temperature. mPEG-OPSS (1.2 mg) was dissolved in 0.012 ml of H<sub>2</sub>O to make a 10% mPEG-OPSS solution. The mPEG-OPSS solution was quickly added to 0.5 ml of Factor VIII solution [0.398 mg/ml in 50 mM Histidine, 0.5 M NaCl, 4.0 mM CaCl<sub>2</sub>, 0.1% (w/v) PEG 3,350, 0.01% (w/v) Tween 80, pH 6.7] and mixed well. After 30 minutes of reaction at room temperature, the reaction vial was transferred to a cold room (4 °C). The pH was determined (pH 6.7±0.2). The molar ratio of mPEG-OPSS-10K to protein was 100:1. The final mPEG-OPSS concentration was 2.344 mg/ml, and the final Factor VIII concentration was 0.389 mg/ml. The reaction was allowed to proceed overnight at 4°C on Rotomix (slow speed, Thermolyne).

[0239] The conjugate mixture was purified using gel filtration chromatography. A size exclusion chromatography method was developed for analyzing the reaction mixtures, and the final products. SDS-PAGE analysis was also used for the characterization of the samples. The pegylation results and yields using the mPEG-OPSS reagent were similar to those in Example 7, which employed the mPEG-MAL reagent having a molecular weight of 20K.

### Example 10

#### Conjugation of B-Domain Deleted Factor VIII with mPEG-MAL, 30K

[0240] Prior to conjugation, a buffer exchange for B-domain deleted Factor VIII (Factor VIII) was performed to replace histidine with HEPES.

[0241] mPEG-MAL, 30K, stored at -20K °C under argon, was warmed to ambient temperature. The warmed mPEG-MAL (1.0 mg) was dissolved in 0.010 ml of HEPES buffer [50 mM HEPES, 0.15 M NaCl, 4.0 mM CaCl<sub>2</sub>, 0.01% (w/v) Tween 80, pH 7.0] to make a 10% mPEG-MAL solution. The mPEG-MAL solution was quickly added to 0.5 ml of Factor VIII solution [0.447 mg/ml in 50 mM HEPES, 0.5 M NaCl, 4 mM CaCl<sub>2</sub>, 0.1% (w/v) PEG 3,350,

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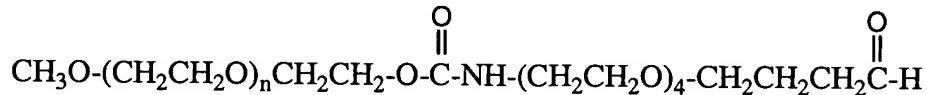
0.01% (w/v) Tween 80, pH 7.0] and mixed well. After 30 minutes of reaction at room temperature, the reaction vial was transferred to a cold room (4 °C), and another 0.010 ml of mPEG-MAL solution was added to the reaction mixture, followed by the addition of three more aliquots of 0.010 ml of mPEG-MAL solution over the course of two hours. The pH was determined (pH 7.0±0.2). The molar ratio of mPEG-MAL to protein was 100:1. The final mPEG-MAL concentration was 9.091 mg/ml, and the final Factor VIII concentration was 0.406 mg/ml. The reaction was allowed to proceed overnight at 4 °C on Rotomix (slow speed, Thermolyne).

[0242] The conjugate mixture was purified using gel filtration chromatography. A size exclusion chromatography method was developed for analyzing the reaction mixtures, and the final products. SDS-PAGE analysis was also used for the characterization of the samples. The PEGylation results and yields using the mPEG-MAL reagent having a molecular weight of 30 K were similar to those in Example 7, which employed the mPEG-MAL reagent having a molecular weight of 20K.

### Example 11

#### Conjugation of B-Domain Deleted Factor VIII with mPEG-Butyr-ALD, 30K

[0243] Prior to conjugation, a buffer exchange for B-domain deleted Factor VIII (Factor VIII) was performed to replace histidine with HEPES.



[0244] mPEG-Butyr-ALD, 30K (shown above), stored at -20 °C under argon, was warmed to ambient temperature. The warmed mPEG-Butyr-ALD (3.8 mg) was dissolved in 0.038 ml of H<sub>2</sub>O to make a 10% mPEG-Butyr-ALD solution. The mPEG-Butry-ALD solution was quickly added to 0.5 ml of Factor VIII solution [0.400 mg/ml in 50 mM HEPES, 0.5 M NaCl, 5 mM CaCl<sub>2</sub>, 0.1% (w/v) PEG 3,350, 0.01% (v/v) Tween 80, pH 7.0] and mixed well. After 15 minutes, 0.060 ml of 10 mM sodium cyanoborohydride solution was added. The pH

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was determined (pH 7.0±0.2). The molar ratio of mPEG-Butyr-ALD to protein was 100:1. The final mPEG-Butyr-ALD concentration was 6.355 mg/ml. The final Factor VIII concentration was 0.334 mg/ml, and the final concentration of NaCNBH<sub>3</sub> was 1.003 mM. The reaction was allowed to proceed for 5 hours at room temperature, and then, overnight at 4 °C on Rotomix (slow speed, Thermolyne).

[0245] The conjugate mixture was purified using gel filtration chromatography. A size exclusion chromatography method was developed for analyzing the reaction mixtures, and the final products. SDS-PAGE analysis was also used for the characterization of the samples. The yield of Factor VIII mono-PEG conjugate was approximately 20%.

### **Example 12**

#### **In-vitro Activity of Exemplary Factor VIII-PEG Conjugates**

[0246] The in-vitro activities of the Factor VIII-PEG conjugates described in Examples 6, 7, and 8 were determined. All of the Factor VIII conjugates tested were bioactive.

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SEQUENCE LISTING

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Mary J. Bossard

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Phe Pro Pro Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val  
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Ala Glu Val Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser  
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Asp Lys Val Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu  
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Tyr Leu Ser His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile  
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Gln Thr Leu His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly  
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Tyr Trp His Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile  
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Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp Tyr Tyr Glu  
705 710 715 720

Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys Asn Asn Ala  
725 730 735

Ile Glu Pro Arg Ser Phe Ser Gln Asn Ser Arg His Pro Ser Thr Arg  
740 745 750

Gln Lys Gln Phe Asn Ala Thr Thr Ile Pro Glu Asn Asp Ile Glu Lys  
755 760 765

Thr Asp Pro Trp Phe Ala His Arg Thr Pro Met Pro Lys Ile Gln Asn  
770 775 780

Val Ser Ser Ser Asp Leu Leu Met Leu Leu Arg Gln Ser Pro Thr Pro  
785 790 795 800

His Gly Leu Ser Leu Ser Asp Leu Gln Glu Ala Lys Tyr Glu Thr Phe  
805 810 815

Ser Asp Asp Pro Ser Pro Gly Ala Ile Asp Ser Asn Asn Ser Leu Ser  
820 825 830

Glu Met Thr His Phe Arg Pro Gln Leu His His Ser Gly Asp Met Val  
835 840 845

Phe Thr Pro Glu Ser Gly Leu Gln Leu Arg Leu Asn Glu Lys Leu Gly  
850 855 860

Thr Thr Ala Ala Thr Glu Leu Lys Lys Leu Asp Phe Lys Val Ser Ser  
865 870 875 880

Thr Ser Asn Asn Leu Ile Ser Thr Ile Pro Ser Asp Asn Leu Ala Ala  
885 890 895

Gly Thr Asp Asn Thr Ser Ser Leu Gly Pro Pro Ser Met Pro Val His  
900 905 910

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Tyr Asp Ser Gln Leu Asp Thr Thr Leu Phe Gly Lys Lys Ser Ser Pro  
915 920 925

Leu Thr Glu Ser Gly Gly Pro Leu Ser Leu Ser Glu Glu Asn Asn Asp  
930 935 940

Ser Lys Leu Leu Glu Ser Gly Leu Met Asn Ser Gln Glu Ser Ser Trp  
945 950 955 960

Gly Lys Asn Val Ser Ser Thr Glu Ser Gly Arg Leu Phe Lys Gly Lys  
965 970 975

Arg Ala His Gly Pro Ala Leu Leu Thr Lys Asp Asn Ala Leu Phe Lys  
980 985 990

Val Ser Ile Ser Leu Leu Lys Thr Asn Lys Thr Ser Asn Asn Ser Ala  
995 1000 1005

Thr Asn Arg Lys Thr His Ile Asp Gly Pro Ser Leu Leu Ile Glu  
1010 1015 1020

Asn Ser Pro Ser Val Trp Gln Asn Ile Leu Glu Ser Asp Thr Glu  
1025 1030 1035

Phe Lys Lys Val Thr Pro Leu Ile His Asp Arg Met Leu Met Asp  
1040 1045 1050

Lys Asn Ala Thr Ala Leu Arg Leu Asn His Met Ser Asn Lys Thr  
1055 1060 1065

Thr Ser Ser Lys Asn Met Glu Met Val Gln Gln Lys Lys Glu Gly  
1070 1075 1080

Pro Ile Pro Pro Asp Ala Gln Asn Pro Asp Met Ser Phe Phe Lys  
1085 1090 1095

Met Leu Phe Leu Pro Glu Ser Ala Arg Trp Ile Gln Arg Thr His  
1100 1105 1110

Gly Lys Asn Ser Leu Asn Ser Gly Gln Gly Pro Ser Pro Lys Gln  
1115 1120 1125

Leu Val Ser Leu Gly Pro Glu Lys Ser Val Glu Gly Gln Asn Phe  
1130 1135 1140

Leu Ser Glu Lys Asn Lys Val Val Val Gly Lys Gly Glu Phe Thr  
1145 1150 1155

Lys Asp Val Gly Leu Lys Glu Met Val Phe Pro Ser Ser Arg Asn  
1160 1165 1170

Leu Phe Leu Thr Asn Leu Asp Asn Leu His Glu Asn Asn Thr His  
1175 1180 1185

Asn Gln Glu Lys Lys Ile Gln Glu Glu Ile Glu Lys Lys Glu Thr  
1190 1195 1200

Leu Ile Gln Glu Asn Val Val Leu Pro Gln Ile His Thr Val Thr  
1205 1210 1215

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Gly Thr Lys Asn Phe Met Lys Asn Leu Phe Leu Leu Ser Thr Arg  
1220 1225 1230

Gln Asn Val Glu Gly Ser Tyr Asp Gly Ala Tyr Ala Pro Val Leu  
1235 1240 1245

Gln Asp Phe Arg Ser Leu Asn Asp Ser Thr Asn Arg Thr Lys Lys  
1250 1255 1260

His Thr Ala His Phe Ser Lys Lys Gly Glu Glu Asn Leu Glu  
1265 1270 1275

Gly Leu Gly Asn Gln Thr Lys Gln Ile Val Glu Lys Tyr Ala Cys  
1280 1285 1290

Thr Thr Arg Ile Ser Pro Asn Thr Ser Gln Gln Asn Phe Val Thr  
1295 1300 1305

Gln Arg Ser Lys Arg Ala Leu Lys Gln Phe Arg Leu Pro Leu Glu  
1310 1315 1320

Glu Thr Glu Leu Glu Lys Arg Ile Ile Val Asp Asp Thr Ser Thr  
1325 1330 1335

Gln Trp Ser Lys Asn Met Lys His Leu Thr Pro Ser Thr Leu Thr  
1340 1345 1350

Gln Ile Asp Tyr Asn Glu Lys Glu Lys Gly Ala Ile Thr Gln Ser  
1355 1360 1365

Pro Leu Ser Asp Cys Leu Thr Arg Ser His Ser Ile Pro Gln Ala  
1370 1375 1380

Asn Arg Ser Pro Leu Pro Ile Ala Lys Val Ser Ser Phe Pro Ser  
1385 1390 1395

Ile Arg Pro Ile Tyr Leu Thr Arg Val Leu Phe Gln Asp Asn Ser  
1400 1405 1410

Ser His Leu Pro Ala Ala Ser Tyr Arg Lys Lys Asp Ser Gly Val  
1415 1420 1425

Gln Glu Ser Ser His Phe Leu Gln Gly Ala Lys Lys Asn Asn Leu  
1430 1435 1440

Ser Leu Ala Ile Leu Thr Leu Glu Met Thr Gly Asp Gln Arg Glu  
1445 1450 1455

Val Gly Ser Leu Gly Thr Ser Ala Thr Asn Ser Val Thr Tyr Lys  
1460 1465 1470

Lys Val Glu Asn Thr Val Leu Pro Lys Pro Asp Leu Pro Lys Thr  
1475 1480 1485

Ser Gly Lys Val Glu Leu Leu Pro Lys Val His Ile Tyr Gln Lys  
1490 1495 1500

Asp Leu Phe Pro Thr Glu Thr Ser Asn Gly Ser Pro Gly His Leu  
1505 1510 1515

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Asp Leu Val Glu Gly Ser Leu Leu Gln Gly Thr Glu Gly Ala Ile  
1520 1525 1530

Lys Trp Asn Glu Ala Asn Arg Pro Gly Lys Val Pro Phe Leu Arg  
1535 1540 1545

Val Ala Thr Glu Ser Ser Ala Lys Thr Pro Ser Lys Leu Leu Asp  
1550 1555 1560

Pro Leu Ala Trp Asp Asn His Tyr Gly Thr Gln Ile Pro Lys Glu  
1565 1570 1575

Glu Trp Lys Ser Gln Glu Lys Ser Pro Glu Lys Thr Ala Phe Lys  
1580 1585 1590

Lys Lys Asp Thr Ile Leu Ser Leu Asn Ala Cys Glu Ser Asn His  
1595 1600 1605

Ala Ile Ala Ala Ile Asn Glu Gly Gln Asn Lys Pro Glu Ile Glu  
1610 1615 1620

Val Thr Trp Ala Lys Gln Gly Arg Thr Glu Arg Leu Cys Ser Gln  
1625 1630 1635

Asn Pro Pro Val Leu Lys Arg His Gln Arg Glu Ile Thr Arg Thr  
1640 1645 1650

Thr Leu Gln Ser Asp Gln Glu Glu Ile Asp Tyr Asp Asp Thr Ile  
1655 1660 1665

Ser Val Glu Met Lys Lys Glu Asp Phe Asp Ile Tyr Asp Glu Asp  
1670 1675 1680

Glu Asn Gln Ser Pro Arg Ser Phe Gln Lys Lys Thr Arg His Tyr  
1685 1690 1695

Phe Ile Ala Ala Val Glu Arg Leu Trp Asp Tyr Gly Met Ser Ser  
1700 1705 1710

Ser Pro His Val Leu Arg Asn Arg Ala Gln Ser Gly Ser Val Pro  
1715 1720 1725

Gln Phe Lys Lys Val Val Phe Gln Glu Phe Thr Asp Gly Ser Phe  
1730 1735 1740

Thr Gln Pro Leu Tyr Arg Gly Glu Leu Asn Glu His Leu Gly Leu  
1745 1750 1755

Leu Gly Pro Tyr Ile Arg Ala Glu Val Glu Asp Asn Ile Met Val  
1760 1765 1770

Thr Phe Arg Asn Gln Ala Ser Arg Pro Tyr Ser Phe Tyr Ser Ser  
1775 1780 1785

Leu Ile Ser Tyr Glu Glu Asp Gln Arg Gln Gly Ala Glu Pro Arg  
1790 1795 1800

Lys Asn Phe Val Lys Pro Asn Glu Thr Lys Thr Tyr Phe Trp Lys  
1805 1810 1815

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Val Gln His His Met Ala Pro Thr Lys Asp Glu Phe Asp Cys Lys  
1820 1825 1830

Ala Trp Ala Tyr Phe Ser Asp Val Asp Leu Glu Lys Asp Val His  
1835 1840 1845

Ser Gly Leu Ile Gly Pro Leu Leu Val Cys His Thr Asn Thr Leu  
1850 1855 1860

Asn Pro Ala His Gly Arg Gln Val Thr Val Gln Glu Phe Ala Leu  
1865 1870 1875

Phe Phe Thr Ile Phe Asp Glu Thr Lys Ser Trp Tyr Phe Thr Glu  
1880 1885 1890

Asn Met Glu Arg Asn Cys Arg Ala Pro Cys Asn Ile Gln Met Glu  
1895 1900 1905

Asp Pro Thr Phe Lys Glu Asn Tyr Arg Phe His Ala Ile Asn Gly  
1910 1915 1920

Tyr Ile Met Asp Thr Leu Pro Gly Leu Val Met Ala Gln Asp Gln  
1925 1930 1935

Arg Ile Arg Trp Tyr Leu Leu Ser Met Gly Ser Asn Glu Asn Ile  
1940 1945 1950

His Ser Ile His Phe Ser Gly His Val Phe Thr Val Arg Lys Lys  
1955 1960 1965

Glu Glu Tyr Lys Met Ala Leu Tyr Asn Leu Tyr Pro Gly Val Phe  
1970 1975 1980

Glu Thr Val Glu Met Leu Pro Ser Lys Ala Gly Ile Trp Arg Val  
1985 1990 1995

Glu Cys Leu Ile Gly Glu His Leu His Ala Gly Met Ser Thr Leu  
2000 2005 2010

Phe Leu Val Tyr Ser Asn Lys Cys Gln Thr Pro Leu Gly Met Ala  
2015 2020 2025

Ser Gly His Ile Arg Asp Phe Gln Ile Thr Ala Ser Gly Gln Tyr  
2030 2035 2040

Gly Gln Trp Ala Pro Lys Leu Ala Arg Leu His Tyr Ser Gly Ser  
2045 2050 2055

Ile Asn Ala Trp Ser Thr Lys Glu Pro Phe Ser Trp Ile Lys Val  
2060 2065 2070

Asp Leu Leu Ala Pro Met Ile Ile His Gly Ile Lys Thr Gln Gly  
2075 2080 2085

Ala Arg Gln Lys Phe Ser Ser Leu Tyr Ile Ser Gln Phe Ile Ile  
2090 2095 2100

Met Tyr Ser Leu Asp Gly Lys Lys Trp Gln Thr Tyr Arg Gly Asn  
2105 2110 2115

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Ser Thr Gly Thr Leu Met Val Phe Phe Gly Asn Val Asp Ser Ser  
2120 2125 2130

Gly Ile Lys His Asn Ile Phe Asn Pro Pro Ile Ile Ala Arg Tyr  
2135 2140 2145

Ile Arg Leu His Pro Thr His Tyr Ser Ile Arg Ser Thr Leu Arg  
2150 2155 2160

Met Glu Leu Met Gly Cys Asp Leu Asn Ser Cys Ser Met Pro Leu  
2165 2170 2175

Gly Met Glu Ser Lys Ala Ile Ser Asp Ala Gln Ile Thr Ala Ser  
2180 2185 2190

Ser Tyr Phe Thr Asn Met Phe Ala Thr Trp Ser Pro Ser Lys Ala  
2195 2200 2205

Arg Leu His Leu Gln Gly Arg Ser Asn Ala Trp Arg Pro Gln Val  
2210 2215 2220

Asn Asn Pro Lys Glu Trp Leu Gln Val Asp Phe Gln Lys Thr Met  
2225 2230 2235

Lys Val Thr Gly Val Thr Thr Gln Gly Val Lys Ser Leu Leu Thr  
2240 2245 2250

Ser Met Tyr Val Lys Glu Phe Leu Ile Ser Ser Ser Gln Asp Gly  
2255 2260 2265

His Gln Trp Thr Leu Phe Phe Gln Asn Gly Lys Val Lys Val Phe  
2270 2275 2280

Gln Gly Asn Gln Asp Ser Phe Thr Pro Val Val Asn Ser Leu Asp  
2285 2290 2295

Pro Pro Leu Leu Thr Arg Tyr Leu Arg Ile His Pro Gln Ser Trp  
2300 2305 2310

Val His Gln Ile Ala Leu Arg Met Glu Val Leu Gly Cys Glu Ala  
2315 2320 2325

Gln Asp Leu Tyr  
2330